Charles University in Prague

Faculty of Science

Department of Zoology

Bc. Karolína Klinovská

The Molecular Mechanisms of Sperm-Egg Fusion in Mouse

Molekulární mechanismy fúze spermie a vajíčka u myši

Master´s Thesis

Supervisor: RNDr. Kateřina Hortová, Ph.D

Consultant: RNDr. Nataša Šebková

Prague 2013

Prohlašuji, že jsem tuto práci vypracovala samostatně s použitím citované literatury, pod vedením RNDr. Kateřiny Hortové, PhD.

V Praze 15.8.2013 .....

Bc. Karolína Klinovská

Acknowledgments

Foremost, I would love to express my gratitude to my supervisor Katka Hortová, for her patience, useful comments, friendly motivation and for all the time she has given me during my study. There is no one better a notorious last-moment person could hope for.
I also want to thank Prof. Harry Moore from The University of Sheffield for the initial idea and experiments that have led to this project, material provided and for inspiring conversations over a coffee.
My thanks also go to Nataša Šebková, for endless hours of discussions and making me laugh anytime I needed it, to Martina Černá for introducing me to the methodology and for putting up with all my questions and problems, and to Lenka Bulvová for her undying optimism and generally being the best lab technician possible.
Finally, I would love to thank my amazing colleague Bára Dudková for her support, understanding and all the fun we have had during the hours, days, and nights spent with our favourite little cells. Of course, for the inspiring and helpful atmosphere I am extremely thankful to everybody in our Biodiversity Research Group. A special acknowledgment also belongs to Tereza Voralová from IAPG AS CR for the provided antibodies.
Last but certainly not least, I thank my unparalleled man simply for everything, whole of my amazing family for continuous support, all the laughs, love and home with warm dinner anytime, and to my great friends, who had to go through my endless biological monologues without understanding a word of it – guys, you rock.

Abstract

The mechanism of gamete fusion remains largely unknown on molecular level despite its indisputable significance. A few molecules requisite for membrane interaction are known, among them IZUMO1 on sperm and tetraspanin CD9 on egg. A concept of a large multitprotein complex on both membranes forming a fusion machinery is recently emerging. CD9 is expected to play a role in organizing egg membrane order and to interact laterally with other factors. On the other hand, IZUMO1 contains one immunoglobulin (Ig) domain, which is known for its interaction with various molecules and thus is expected to have a binding partner on the egg membrane. Ig domains are known for their involvement in interactions with Fc receptors and Fc receptor-like molecules, which makes these a potential partner for sperm in gamete binding and fusion.

Recent experiments identified Fc receptor-like 3 as a candidate binding partner for sperm by one-bead one-compound assay. The aim of presented study was to assess the localization of FCRL3 in mouse eggs, together with its potential co-localization with IZUMO1 on complexes of zona-free eggs with bound sperm. In this study, FCRL3 molecule has been found across the cytoplasm, possibly in the intracytoplasmic membrane compartments of the cell, as well as on the egg membrane. Its presence on the surface of the membrane suggests it can represent a receptor for sperm binding.

The primal requirement for direct interaction of molecules is their close proximity, thus FCRL3 and IZUMO1 localization was assessed at first by immunofluorescent co-localization assay. The method detected overlapping fluorescent signals of anti-FCRL3 and anti-IZUMO1 antibodies on sperm-egg complex *in vitro* indicating a co-presence of the proteins. This finding was further investigated by Proximity ligation assay, a novel method detecting proteins closer than 40 nm, thus in distance enabling their direct interaction. The encountered problems with negative control and its analysis prevented the assessment of the close proximity of FCRL3 and IZUMO1 by this assay.

Our results show that FCRL3 localizes on the egg membrane, indicating it might function in an unknown way in fusion process. The position of FCRL3 co-localizes with the site of sperm attachment and IZUMO1, thus, the role of FCRL3 as a binding partner for IZUMO1 cannot be ruled out and its potential function as a critical player in gamete fusion machinery needs to be investigated further.

Key words: gamete fusion, IZUMO1, FRCL-3

Abstrakt

Molekulární mechanismus fúze gamet zůstává i přes svoji klíčovou roli v oplození z velké části neobjasněn. Bylo popsáno několik molekul, mezi nimi spermatický protein IZUMO1 a tetraspanin CD9 na vajíčku, které jsou nutné pro průběh interakce a fúze gamet u myši. Ze známých dat se v posledních letech formuje koncept velkého multiproteinového komplexu na obou membránách, společně tvořících fúzní aparát. Předpokládá se, že tetraspanin CD9 hraje roli v organizaci a udržování membránových struktur díky laterálním interakcím s ostatními membránovými proteiny. Naproti tomu se očekává, že IZUMO1, obsahující imunoglobulinovou doménu (Ig), má na povrchu vajíčka svůj vazebný receptor. Ig doména je známá pro své interakce s jinými protein, mezi nimi především Fc receptory, které by tak mohly představovat potenciální vazebné proteiny na povrchu vajíčka.

Nedávné experimenty identifikovaly molekulu Fc receptor-like 3 (FCRL-3) jako kandidátní vazebný faktor vajíčka pomocí metody one-bead one-compound. Cílem této práce bylo určit lokalizaci FCRL3 na myších vajíčcích a případnou kolokalizaci s proteinem IZUMO1 na komplexu vajíčka s navázanými spermiemi, nutnou pro jejich možnou interakci. Pomocí fluorescenčního značení byl FCRL-3 identifikován jak v cytoplazmě, tak na povrchu plazmatické membrány vajíčka. Přírомnost molekuly na membráně naznačuje možnost, že hraje roli v interakci spermie a vajíčka.

Pro přímou interakci molekul je nezbytná jejich prostorová blízkost, která byla zhodnocena kolokalizačním pokusem. Tato metoda zaznamenává překrývající se signál protilátek proti FCRL3 a IZUMO1 na vajíčku s navázanými spermiemi. Společný výskyt signálu naznačuje jejich možnou interakci, která byla dále zkoumána metodou Proximity ligation assay, detekující molekuly ve vzdálensti menší nez 40 nm. Nespecifické výsledky negativní kontroly vyloučily možnost analýzy této metody, proto nebylo možné určit potenciální těsnou blízkost FCRL3 a IZUMO1.

Výsledky této práce, ukazující lokalizaci FCRL3 na membráně myšího vajíčka, představují nezbytnou podmínku možné receptorové funkce tohoto protein ve vazebném či fúzním aparátu vajíčka. Výskyt FCRL3 kolokalizuje s místem membránového kontaktu se spermií a s výskytem proteinu IZUMO1, což naznačuje jejich možnou interakci. Role FCRL3 ve vazebném či fúzním aparátu tak nemůže být vyloučena.

Klíčová slova: fúze gamet, IZUMO1, FCRL-3

List of abbreviations

AR	acrosome reaction
BSA	bovine serum albumin
DAPI	4´,6 – diaminido-2-phenylindole
DIC	differential interference contrast microscopy imaging
DMSO	dimethyl sulfoxide
FCRL	Fc receptor-like molecule
FGFR2	fibroblast growth factor receptor
FITC	fluorescein isothiocyanate
GPI	glycophosphatidylinositol
Gp41	glycoprotein 41
Ig	immunoglobulin
IU	international unit
NCAM	neural cell adhesion molecule
PBS	phosphate buffered saline
PE	phycoerythrin
PFA	paraformaldehyde
PLA	proximity ligation assay
SNARE	soluble NSF attachment protein receptor mediating vesicle - membrane fusion
SPESP1	sperm equatorial segment protein 1
TEM	tetraspanin enriched domains
ZP	*zona pellucida*

Table of contents

1. Introduction

Fertilization is a multistep and complex process culminating in merger of gamete membranes, cytoplasmic unity and fusion of genomes, initiating the development of a new individual. Even though membrane fusion is a key event in this process, there is still very little known about its mechanism or molecules involved. The fusion shows less distinct species-specificity than do the preceding steps in fertilization, like *zona pellucida*-sperm interaction (Yanagimachi, 1977), which suggests that the mechanism and molecules involved in membrane fusion are more conserved and findings can be applied generally to an extent. During recent years, effort has been made towards the identification of the molecular players and their function, and several molecules have been found to be essential or nearly essential on the egg or the sperm side. Although the concept of multiprotein complexes on both membranes has been accepted in recent years, there are no known molecules of direct interaction in binding and fusion of sperm and egg in mammals.

The only truly essential player on the sperm side known to date is protein IZUMO1. Its knock-out is completely infertile due to impaired fusion (Inoue *et al*., 2005). Several proteins have been found to interact laterally with IZUMO1 in the membrane (Ellerman *et al*., 2009), but as the protein contains an immunoglobulin domain in its extracellular part, it is expected to interact with an unknown binding protein on the egg´s surface.

Common partners for immunoglobulin domain are Fc receptors, well documented in immune system. The mouse sperm is capable of fusion with other cell types than an egg, specifically with B-lymphocytes (Ashide and Scotfield, 1987). These cells are well known for expression of various types of molecules with Fc domains, especially Fc receptor-like (FCRL) molecules. These findings led professor Harry Moore from the University of Sheffield to the experiment of one-bead one-compound assay with various FCRL molecules. The molecule identified by this method as a potential binding partner for sperm was Fc receptor like 3 (FCRL3) (unpublished).

FCRL3 is a transmembrane protein expected to localise in various compartments´ membranes as well as on the plasmatic membrane. If the molecule is detectable on the surface of an egg, it may represent a receptor for gamete binding. Therefore a detection of its exact localization on mouse egg constitutes the first step in assessing whether this molecule may be the long sought after partner of IZUMO1.

The main requirement for direct interaction of two molecules is their close proximity to each other. The primal investigation comprise of immunofluorescent co-localization of the signals of FCRL3 and IZUMO1 on complex of an egg with bound sperm. This way, if the signals do not overlap, the hypothesis about the direct interaction of the studied molecules can be negated. The proximity can also be assessed by more sensitive method, Proximity ligation assay, detecting a single protein-protein interaction by a distinct fluorescent spot only when these localize closer than 40 nm from each other. This distance is considered essential for a direct interaction of two proteins.

Localization of FCRL3 on egg membrane, its potential co-localization with IZUMO1 on sperm and their close proximity during sperm-egg binding represents a pilot study for a potentially first discovered molecule playing role in direct interaction of membranes in mouse gamete fusion. The fusion machinery on both membranes appears to be very complex, and every newly discovered factor adds to our understanding of this vital process.

2. Aims of the thesis

The main aim of presented work was to localize and monitor the distribution and potential interaction of known fusion-essential protein on sperm (IZUMO1) and its candidate binding partner FCRL-3 on egg using C57Bl/6 inbred mice gametes *in vitro*:

1) **Optimization of FCRL-3 monoclonal antibody on ovulated zona-free mouse eggs**
2) **Monitoring of FCRL-3 distribution in the egg**

   Occurrence of FCRL-3 in the cytoplasmic membranes and the confirmation of FCRL-3 localization on the extracellular side of egg plasma membrane as a potential receptor
3) **Immunofluorescent co-localization of FCRL-3 and IZUMO1**

   Parallel fluorescent staining of FCRL-3 on egg and sperm IZUMO1 on the unfixed complex of zona-free egg with attached sperm
4) **Assessing the possible interaction of FCRL-3 and IZUMO1 by Proximity ligation assay in situ (PLA)**

   Confirmation of co-localization results by PLA, which detects proteins in immediate vicinity that enables their direct interaction

3. The literature review

3.1. Fusion as a crucial biological event

Membrane fusion is one the most fundamental processes in multicellular organisms, enabling wide range of actions, such as sexual reproduction, vesicular trafficking, immune reactions and neurotransmission. This study concentrates on molecules participating in gamete fusion, where the knowledge of general mechanism in different context might be truly helpful. It has been extensively studied for many years, yet the overall picture of the mechanism is far from complete. The mixing of two phospholipid bilayers occurs in 3 contexts - virus-cell fusion, intracellular vesicle fusion and cell-cell fusion. Although virus-cell and vesicle fusion are relatively well examined (reviewed by Jahn *et al*., 2003), cell-cell fusion mechanism remains surprisingly unknown despite its physiological importance. It is expected that mechanistic concepts of all the three are at least partly shared despite the differences between conditions in which they take place.

There have been many attempts to divide the fusion process into stages, to make the comparisons easier. They vary among each other, but generally it can be said that the process requires gaining of cellular competence to fuse, membranes recognition and commitment, induction and activation of the fusion machinery, membrane apposition and finally lipid bilayers mixing (Stein *et al*., 2004; Aguilar *et al*., 2013). In different systems the target specificity is ensured in different stages - either membrane recognition, or induction (sperm unable to fuse with an egg still binds to it, Inoue *et al*., 2005). The conceptual framework in the field assumes specific proteins on the membrane that are essential for fusion to be either involved in attachment, or mediate merging of the cell membranes (fusogens *per se*) (Aguilar *et al*., 2013).

Regarding the attachment, in many virus-cell fusion systems multiple proteins participate in a single virus-cell attachment event, facilitating a complex interaction occurring in a limited time frame. The machinery often comprise of adhesion domains or carbohydrate moieties on membrane proteins (Dimitrov, 2004). It is predicted that cell-cell attachment proteins would share these characteristics, as per instance the immunoglobulin (Ig) superfamily members involved in Drosophila myoblast fusion do (Taylor, 2002). Each of these proteins contains several Ig-like domains, which are well defined cell-cell adhesive domains.

The lone process of fusion is mediated by cell fusion proteins (fusogens) that bring the membranes closer together and mediate the mixing of bilayers. Upon receiving the induction signal, these molecules, linking the intermembrane space, irreversibly fold back on themselves in a hing-like motion and draw the membranes very close together, enabling the two lipid bilayers to mix (Oren-Suissa and Podbilewicz, 2007). In well investigated systems several molecules have been identified as bona fide fusogens, i.e. in Gp41 in viral fusion (Chan and Kim, 1998; Harrison, 2008) or SNARE proteins in synaptic vesicles fusion (Weber *et al*., 1998; Rizo and Rosenmund, 2008).

In the mammals, there has been reported one family of well defined fusogens named syncitins. This family includes proteins derived from endogenous retroviruses related to the HIV Gp41 envelope glycoprotein, and function during formation of syncitial trophoblast essential for mouse placentation (Dupressoir *et al*., 2005). They were proved to be *bona fide* fusogens, as they induce cell-cell fusion in different cell lines in a receptor-dependent manner, with disulfide bridge-forming motifs essential for their fusogenic activities (Chen *et al*., 2008).

The fusogens in other systems and species are being intensively hunted. The difficulties in the field are mainly caused by the fact that based on work on viral fusogens, the overall structure rather than the primary sequence is conserved (White *et al*., 2008), and the fact that cell-cell fusion is believed to be restricted to specific cell types, often complicated to work with.

3.2. Interaction of gametes culminating in fusion

Despite the amazing variety in organisms, it still takes two to tango in sexual reproduction – sperm and egg meet and fuse to ensure mixing of genetic material and development of a new unique individual. On the way, gametes (especially the sperm) undergo series of events changing their morphology, structure and functionality, only to allow them to recognize each other and fuse. Eggs acquire the competence to fuse with sperm during oogenesis and are capable of fusion since they are at least 20 μm in diameter while still arrested in prophase of the first meiotic division (Zuccoti *et al*., 1994). Sperm experience a great transformation to become fertilization-competent during its passage through female reproductive tract, with capacitation and acrosome reaction changing its motility, physiology and molecular membrane structure without which the sperm fails to pass on its precious genetic cargo.

Capacitation is the first step in rendering the sperm capable of interaction with an egg. It is basically a functional maturation of the sperm, involving increase in membrane fluidity due to cholesterol efflux, changes in sperm membrane potential, increased tyrosine phosphorylation and induction of hyperactivation (Tash and Means, 1983). It is followed by acrosome reaction - fusion of plasma and outer acrosome membranes, exposing the inner acrosome membrane and releasing the acrosomal content. It can be triggered by multiple factors – contact with ZP, progesterone concentration or even spontaneously – suggesting that timing of this essential process is redundant and the different time of onset in different population of sperm may play role in sperm competition (Veselá, 2012). The exact combination of causes and effects is not clear, however, it is well known that the acrosome reacted sperm penetrates ZP, enters the perivitelline space and is able of fuse with oolema. The sperm cells that did not undergo this process bind to an egg, but are incapable of fusion with it, which indicates that essential factors on the sperm membrane are either exposed or modificated by this massive exocytosis (Yanagimachi and Noda, 1970).

The fusion site is specific on both gametes, which leads us to believe there are topologically unique protein populations or lipid organization sites with distinct membrane morphology required for fusion (Stein *et al*., 2004). The sperm membrane overlying the acrosome, that does not take part in the acrosome reaction, is called the equatorial region, and the sperm-egg fusion is long believed to be initiated in this region (Yanagimachi, 1988). The surface of rodent egg plasma membrane can be divided into two parts: the microvillar-free smooth region, which overlays the meiotic spindle, and the microvillar protrusions-rich region, covering the rest of the egg, forming a dome shaped structure antipodal to eccentric nuclei. Gamete fusion occurs predominantly (Johnson *et al*., 1975) or exclusively (Ebensperger and Barros, 1984) in the microvillar-rich region.

When the two membranes are merged, creating a new zygote membrane, the inner acrosomal membrane, forming anterior of the sperm head, is excluded from the merger. It fuses with a small patch of oolema and forms a separate detached hybrid vesicle in the cytoplasm, in a process described as pseudo-phagocytotic-like (Primakoff and Myles, 2007).

Despite a great effort, the molecular basis of the gamete interaction is still poorly understood. During the course of research history there have been many shifts in paradigms, completely dismissing the previous view and building a new one on recent discoveries. Naturally, this was made possible by the advances in technologies. Monoclonal antibodies, *in vitro* fertilization and particularly knock-out organisms with no expression of a specific molecule are methods which changed our understanding the most, and now represent the

grounding technologies in the field. For example method of producing knock-out mice strains has shaken the existing belief that integrins are the most important adhesion and fusion molecules on egg by proving that not one up to date known integrin is essential for fusion which occurs even when they are not expressed at all (He *et al*., 2003). Thanks to this method, the only truly essential fusion factors known so far, IZUMO on sperm and CD9 on egg, were discovered as well (see below).

3.3. Identified players in gamete fusion in mouse

In the last couple of years, the completely new picture of fusion machinery is emerging thanks to the new methods and protracted unravelling of the factors step by step. Even though our comprehension is far from complete, some molecules are established as either essential, nearly essential or associated with the essential factors, forming an intricate and partly redundant system securing the process of fertilization.

3.3.1. Essential molecules on the sperm side

3.3.1.1. SLLP1

In 2005, Herrero *et al*. discovered mouse sperm lyzozyme-like acrosomal protein that relocates into equatorial segment after acrosome reaction. It was proposed to play a role in gamete interaction, which was proved by *in vitro* fertilization assay, where the specific antibody against SLLP1 blocked both fertilization and binding. Receptor sites for this protein are found in the microvillar region of the egg and in the perivitelline space, which is in agreement with sites of CD9 (Ohnami *et al*., 2012). The binding partner of SLLP1 was however found to be SAS1B, specific oolemal metalloprotease (Sachdev *et al*., 2012).

3.3.1.2. IZUMO1

At first, the monoclonal anti-mouse antibody against an unknown antigen on sperm surface , inhibiting the fusion process both *in vivo* and *in vitro,* was characterized through screening of anti-sperm monoclonal antibodies (Okabe M *et al*., 1988). This antibody was named OBF13 and its corresponding antigen was not identified for many years. In 2005, Inoue *et*

al. determined this protein by 2D gel electrophoresis, immunoblotting and the liquid chromatography-tandem mass spectrometry analysis, and named it IZUMO after a Japanese shrine dedicated to marriage. The question whether IZUMO1 functions as a truly essential factor in fertilization could have been answered only by generating Izumo1-deficient mice by homologous recombination. Izumo$^{-/-}$ mice were found healthy and without any developmental abnormalities, but as expected the males were sterile despite normal mating behaviour. The sperm penetrated the ZP without any problems but failed to fuse with eggs, resulting in accumulation of sperm in the perivitelline space of the egg. Izumo$^{-/-}$ sperm defect is limited to the fusion ability, as proven by an injection of the sperm into wild type eggs, which resulted in normal implantation, to term development of the offsprings in normal ratio and their ability to reproduce.

IZUMO was found out to belong to immunoglobulin superfamily of type I membrane proteins with one extracellular immunoglobulin (Ig) domain and one N-terminal domain. The superfamily consists of four proteins, coded with numbers 1 to 4, showing a significant homology in N-terminal domain, hence known as 'IZUMO domain'. IZUMO1 (originally described by Inoue's group), 2 and 3 are transmembrane proteins expressed only in the testis, whereas IZUMO4 is soluble and expressed in the testis and other tissues (Ellerman *et al*., 2009).

The fusion-indispensable IZUMO1 is not expressed in the same place on the sperm during its course through female reproductive tract and fertilization process, especially during acrosome reaction. Sperm can be divided into three groups depending on their acrosomal reaction state and IZUMO1 staining pattern - acrosomal cap, equatorial and whole head. IZUMO1 relocates during AR from the anterior part of the sperm head to the sites where the fusion would take place. Since it is said that sperm launch fusion with an egg at the equatorial segment, either equatorial or whole-head type IZUMO1 can contribute to sperm-egg fusion.

Although IZUMO1 is the only known essential factor in the sperm, and is often described as primary fusogen of the sperm side, its only functional domain is the immunoglobulin one. The molecule lacks any fusogenic peptide domain or domain resembling fusogenic peptides in other systems like viral penetration or intracellular vesicular trafficking. Therefore, it seems probable that IZUMO1 interacts with associated proteins that directly facilitate the fusion process in a multiprotein complex on the sperm membrane (Inoue *et al*., 2011). Ongoing research is directed at search for these associated proteins, as it is predicted that the functional domain would at least share characteristics with other fusogens *per se* and the

factor is expected to be essential, and therefore would block the fusion in knock-out systems. Nevertheless, the Ig proteins are well known to function as antigen receptor, co-receptor and adhesion molecule through interactions. It is therefore expected that IZUMO1 interacts directly with some molecule on the oolema, but this ligand has yet to be identified, as well as the precise function of IZUMO1 protein, whether it is a regulator of a fusogen, or/and an adhesion molecule.

3.3.1.3. Proteins associated with IZUMO1

The previous works lead to an easy assumption that IZUMO1 is a sperm fusogen. However, the protein lacks any fusogenic peptide part or 'SNARE' like structure, as would be expected by finding of works on other fusion systems in mammal cells. This opens the possibility that IZUMO1 might be an essential factor in a protein complex that might contain or modulate other fusion molecules. Ellerman *et al.* (2009) showed that IZUMO1 forms complexes with other proteins on the sperm surface and suggested that its N-terminal domain possesses the ability to form dimers. This supports the hypothesis of the protein being involved in organizing or stabilizing a multiprotein complex essential for the function of the membrane fusion machinery. The current approach assumes a direct link of IZUMO1 with an unknown protein on egg's surface, even though this might prove not to be fusogen *sensu stricto.*

3.3.2. Egg's point of view - the known molecules

Throughout the long studies of gamete interaction, a few families of proteins have been proposed as binding or fusogenic players on the egg's side. Many of the previously described proteins were proven false leads by current knock-out mouse lines, nevertheless, a couple of candidates are emerging to shed a light on the fusogenic machinery on the egg surface.

3.3.2.1. Tetraspanin family

The tetraspanin family consists of small (20-50 kD) transmembrane proteins that contain four transmembrane domains (which gave the family its name) with two extracellular loops and have wide tissue distribution. Through the larger extracellular loop, the molecule directly or indirectly *cis*-interacts with other membrane proteins as immunoglobulins (Ig),

signalling enzymes and integrins (Berditchevski, 2004), and mediate the assembly of structural and functional units called the tetraspanin enriched microdomains (TEMs), analogous to microdomain lipid rafts (Claas *et al*., 2001). Tetraspanin also communicates with cytoskeletal and signalling molecules via intracellular domains (Zoller, 2009). Within TEMs, tetraspanin are believed to be primarily organizers of the network of transmembrane and cytoplasmic effector molecules such as receptors, fusogens and signalling proteins, and modulate their function and thus regulate many processes. Tetraspanin network is used to execute functions that require multiple intermolecular interactions. It has been reported that some tetraspanin molecules may act as receptors, but so far the examples are few (i.e. Farquhar *et al*., 2011). The conservation of tetraspanin genes suggests they appeared early in evolution and perform vital functions.

3.3.2.1.1. CD9

An important member of tetraspanin family is ubiquitously expressed protein CD9. It functions as a regulator of wide range of processes varying from fusion of myoblasts (Tachibana and Hemler, 1999) and monocytes (Takeda *et al*., 2003) to cell signalling and adhesion. As expected from such a versatile tetraspanin protein, it interacts laterally with many other molecules, including immunoglobulins (Glazar and Evans, 2009), other tetraspanins, a subset of integrins, G proteins or other adhesion molecules (Le Naour *et al*., 2006).

At first, it has been reported that antibody against CD9 reduces fertilization rate (Chen *et al*., 1999). The discovery that the protein is nearly irreplaceable for fertilization was made by chance serendipitously and simultaneously in three laboratories during research of the effects of CD9 knock-out on immune system (Miyado *et al*., 2000; Le Naour *et al*., 2000; Kaji *et al*.; 2000). It was found that the knock-out mice are healthy, but females have severely reduced fertility. The defect is fusion related, as the wild type sperm penetrates the *zona pellucida*, binds to oolema but the membranes fail to fuse. The infertility is overcome by intracytoplasmic injection and embryos develop normally. This was a very striking and uncommon knock-out phenotype, as it represents widely expressed protein manifesting very specific, nonredundant effect only in the egg, where other proteins from the same family are also expressed, unable to replace its function.

As CD9 is up to date the only factor known to be almost completely essential on the egg, its exact function has been extensively studied ever since. There have been three theories

proposed about its function – *cis*-interaction with other membrane proteins, trans-interaction with sperm receptor or a role in membrane structure. These hypotheses do not exclude each other and indeed all three can be proven correct.

The second hypothesis about trans-interaction arose from the research of macrophage regulation, where CD9 was found to bind pregnancy specific glycoprotein PSG17, a member of immunoglobulin superfamily (Waterhouse *et al*., 2002). The glycoprotein was found to inhibit fusion if added to zona-free eggs (Ellerman *et al*., 2003), but is not discovered on sperm surface. At least two related glycoprotein from the sub-family are expressed in the sperm, but no evidence for their role in sperm-egg fusion has yet been presented (Primakoff and Myles, 2007). According to new researches, CD9 may play a role rather in sperm-egg binding than fusion. CD9-null eggs show reduced ability for strong sperm adhesion (Jégou *et al*., 2011), and sperm accumulate in the perivitelline space, only transiently binding to the egg surface. This suggests a role in adhesion strengthening (Zhu and Evans, 2002).

The assumption about CD9 role in membrane order was proven true by researches of microvilli organization and morphology in CD9-defficient eggs. Immunofluorescence shows that CD9 is localized to the microvillar-rich region of the egg (Kaji *et al*., 2000), with specifity to protrusions rather than to the planar membrane between them (Runge *et al*., 2006). In knock-out mice, the morphology of microvilli is altered, as these appear shorter, thicker and loosely distributed, with the radius of curvature appearing wider (Runge *et al*., 2006). If we accept the role in membrane order, we can also easily assume that the first hypothesis about cis-interaction is true, as such a significant role in morphology surely demands cooperation with other membrane proteins.

It was also proposed that exosome-like CD9-containing vesicles are secreted from the egg to perivitelline space and transferred to sperm head membrane, thereby conferring fusion competence to the sperm (Miyado *et al*., 2008). However, this experiment could not be reproduced in some independent laboratories and still causes some controversy.

Even though the exact function of CD9 in complex attachment/fusion machinery on egg is not fully understood, it presents one of the best investigated factors and is a starting point for many other hypotheses. There is no evidence for an exact binding partner, as the interaction with IZUMO1, however tempting, was not proven. The exact role of the protein seems to be in organizing the multiprotein complex and morphology of the membrane required for the fusion.

3.3.2.1.2. CD81

A second tetraspanin member, CD81, which resembles CD9 in many features, has been proposed to play role in fusion as it is expressed on the egg surface and often interacts with CD9 in other cell-cell fusion systems (Horváth *et al*., 1998). Deletion of the *Cd81* gene results in less dramatic reduction in fertility, however, the double knock-out for both CD9 and CD81 are completely infertile (Rubinstein *et al*., 2006). There is even an evidence for extracellular role of the CD81, but this has yet to be clarified (Ohnami *et al*., 2012). The receptor function cannot be excluded from hypotheses, as CD81 works as a receptor for hepatitis C viral envelope protein (Pilleri *et al*., 1998) The exact role of this tetraspanin is still under debate (Glazer and Evans, 2009), but it is expected to be part of the fusion machinery with partly redundant, but still important role in the process.

3.3.2.2. Glycosilphospatidylinositol (GPI) anchor

There is a strong evidence for specific requirement of GPI-anchored proteins on egg membrane. At first, GPI-anchored proteins were removed from the egg surface by bacterial-derived PI-PLC, which blocked binding and fusion capability (Coonrod *et al*., 1999). The findings were than confirmed by producing knock-out mice with deletion in *Pig-a* gene, encoding the first enzyme in biosynthesis of GPI, which also resulted in infertile phenotype (Alfieri *et al*., 2003). The connection between tetraspanins, which forms membrane domains, and lipid rafts, sites that contain the GPI, are now investigated, with the working hypothesis of CD9 and GPI-anchored proteins controlling the signalling pathway induced by the adhesion or participating in the appropriate membrane organization together (Lefévre *et al*., 2010).

3.3.2.3. SAS1B

When the first binding factor on the sperm, SLLP1 (Herrero *et al.,* 2005), was found, its partner on the oolema was not known. It was suggested that this protein would confine to microvilli-rich region of the egg surface. The protein was identified and characterized as SAS1B, specifically oolemal metalloprotease in 2012 (Sachdev *et al.*). This protease is concentrated in a dome corresponding to the microvillar region and in perivitelline space,

consistently with the presence of CD9 (Miyado *et al*., 2008). When stained with specific antibody, the protease signal co-localizes with the SLLP1 binding sites on the oolema, indicating interaction. Gene knock-out of the protein in mice showed significantly lowered fertilization rate. SAS1B is the first oocyte specific oolemal metalloprotease yet implicated in gamete binding during fertilization and in duo with SLLP1 it is believed to be one of the binding factors in the attachement-fusion machinery on egg´s surface.

Whether attachment involves the same molecules that participate in the fusion has not been determined, but the fact that sperm with intact acrosome, unable of fusion with oolema, still bind to it, supports the concept of intricate protein complex forming machinery on both membranes, involving fusion, attachment and associated proteins. We currently have no ability to differentiate between physiological attachment of sperm ending in fusion from the artificial sticking that is observed in the *in vitro* assay (Talbot *et al*., 2003).

3.4. FCRL family proteins as potential binding partners of IZUMO1

There has been no binding partner of IZUMO1 on the egg side discovered since the protein was found out to be essential for fusion. As it contains an immunoglobulin domain, which often interacts with Fc domains in immune system, this appears to be a possible direction of the research. The mouse sperm is capable of fusion with other cell types than an egg, specifically with B-lymphocytes (Ashide and Scotfield, 1987). These cells are well known for expression of various types of molecules with Fc domains, especially Fc receptor-like (FCRL) molecules belonging to a large family of preferentially B-lymphocyte co-receptors homologous to the well-known receptors for the Fc portion of immunoglobulin (FCR). These findings led professor Harry Moore from Sheffield University to the experiment of one-bead one-compound assay to sort out the potential candidates from FCRL family for a sperm-binding partner on the oolema.

The experiment consisted of small resin beads with one specific peptide population attached to one bead, together covering various members of FCRL family. The beads are bifunctional, with testing molecules on the outer layer and coding tags in the interior of the bead. After the beads were co-incubated with acrosome reacted sperm, only the beads with bound sperm were collected and the peptides to which they showed such affinity were determined by direct sequencing of the coding tag. One of the most promising candidate binding proteins was found to be FCRL-3 molecule (unpublished).

FCRL-3 is a type I transmembrane protein best examined in human, with immunoreceptor - tyrosine activation motifs and immunoreceptor - tyrosine inhibitory motifs in its cytoplasmic domain, and Fc-homologous extracellular domain (Kochi *et al*., 2005). It is expected to have receptor and tyrosine-regulation activity and to localize in various cell compartments´ membranes. In human, the mutation in FCRL-3 has been associated to several diseases as rheumatoid arthritis, autoimmune thyroid disease and systemic lupus erythematosus (Kochi *et al*., 2005). There is evidence of autoimmune diseases affecting fertility in women, especially autoimmune thyroid disease (Poppe *et al*., 2002) and systemic lupus erythematosus (Friedman and Rutherford, 1956), but there is no data available whether this reduced fertility can be caused by impaired gamete fusion. However, the function may vary among the species and the evaluation of possible role of FCRL3 in mouse gamete interactions cannot be overlooked. As the molecule has been proven to modulate innate signalling in human B cells (Li *et al*., 2013), it may play a similar, or completely unknown role in the mouse fusogenic protein complex.

The presented study examines the possibility of FCRL-3 and IZUMO1 cooperation as a pilot study. It looks into the localization of FCRL-3 molecule in the mouse egg using immunofluoresce staining, collocalization of the molecule with IZUMO1 on egg with bound sperm and potential interaction of these two factors through proximity ligation assay

4. Material and Methods

4.1. Animals used for eggs retrieval and sperm collection

Inbred C57Bl/6 mice (The Jackson Laboratory) housed at 15/9 light dark cycle, light on at 5:00, light off 20:00, maximum 10 females per cage, 2 males per cage

4.2. Materials

4.2.1. Chemicals

M2 Medium (M7167, Sigma)
Paraffin Oil (8904.1, Roth)
Bovine Serum Albumin (A7906, Sigma)
Calcimycin A 23187 (7522, Sigma)
Progesteron (P8783, Sigma)
Vectashield mounting medium with DAPI (H500, Vector)
High purity water (sterile filtered)

4.2.2. Solutions

PBS
100 mL distilled water
1,37mM NaCl
2,7mM KCl
8,7mM Na2HPO4 (for 12 H2O)
1,5mM KH2PO4

Paraformaldehyde (PFA), 30251, Lach-Ner, diluted in PBS
Bovine serum albumin (BSA), A7906-505, Sigma Aldrich, diluted in PBS

4.2.3. Immunofluorescence detection – antibodies

Primary Antibodies

FCRL-3, raised in rabbit against human, HPA 015509 Sigma Aldrich

IZUMO1 (K-14), goat polyclonal IgG against mouse, sc-79539, Santa Cruz Biotechnology

NCAM (H-300) rabbit polyclonal, sc-10735, Santa Cruz Biotechnology

FGFR2 -Bek (N-20) goat polyclonal, sc-31164, Santa Cruz Biotechnology

Secondary Antibodies

Alexa Fluor 488, goat anti-rabbit IgG (H+L), A1108, Invitrogen

Donkey anti-goat IgG-PE, sc-3743, mouse/human adsorbed, Santa Cruz Biotechnology

4.2.4. Solutions for Proximity Ligation Assay Duolink® in Situ (Olink Bioscience)

PLA probe MINUS anti-goat, Art. No. 92006-0030

PLA probe PLUS anti-rabbit, Art. No. 92002-0030

Ligation Reagent (provided by the manufacturer), diluted 1:5 in high purity water

Ligase (1 U/µl) (provided by the manufacturer), diluted 1:40 in Ligation Reagent

Amplification Reagent Red (Art. No. 92008-0030), (provided by the manufacturer) diluted 1:5 in high purity water

Polymerase (10 U/µl) (provided by the manufacturer), diluted 1:80 in Amplification Reagent

Wash Buffer A and Wash Buffer B (a premixes provided by the manufacturer), dissolved in high purity water to a final volume of 1000 ml

4.2.5. Equipment

CO2 incubator (NB-203LC, N-Biotec)

Flowbox (Bio IIA, TELSTAR)

Stereo microscope (S2, Olympus)

Cenrifuge (Mini spin plus, Eppendorf)

Vortex (PV-1, P-LAB)

Motorized Inverted fluorescence microscope with the Cell R system (microscope IX81, Olympus; camera Hamamatsu ORCA C4742-80-12AG)
Humidity chamber
Inverted confocal microscope Leica TCS SP2 with Acousto-Optical Beam Splitter

4.3. Methods

4.3.1. Retrieval of the eggs

Female, 4-6 weeks old C57Bl/6 mice were hormonally stimulated for superovulation with 5IU of follicle-stimulating hormone at 16:00 first day, 5IU of human chorionic gonadotropin at 16:00 on third day, and sacrificed by cervical dislocation 15 hours after. Both ampullas of the fallopian tube were dissected and placed in M2 medium heated to 37 °C. The cumulus oocyte complexes were retrieved from ripped ampullas and places into fresh heated M2. The cumulus cells were removed by incubation in hyaluronidase for 10 min. After washing in M2, the eggs with standard morphology a visible first polar body were separated and *zona pellucida* was removed by Tyrodes solution, three times for 5 minutes, washed in M2 between. The status of zona pellucida was assessed by checking in light field. Now zona-free eggs were checked and allowed to recover in fresh heated M2 medium for 30 minutes.

4.3.2. Immunofluorescent detection of FCRL-3

To find out whether the FCRL-3 antibody binds to the antigens in mouse eggs, immunofluoresce staining was used. For preliminary results, a standard protocol for paraformaldehyde fixed cells was adjusted for work with eggs to determine the dilution factor. Complexes of egg with bound sperm were used to investigate the possible difference in staining pattern after interaction with sperm. The native immunofluorescence staining protocol without fixation was employed for determining whether the epitope is expressed on the extracellular side of egg plasma membrane. The eggs are very fragile cells and usually do not withstand the osmotic pressure during time demanding labelling process. As the sperm attachment seems to stabilize the membrane, the detection on unfixed eggs was also carried out on complexes of eggs with bound sperm.

4.3.2.1. Immunostaining on fixed eggs

- ***Fixation*** - The zona-free eggs were fixed with 3,7% paraformaldehyde for 15 min in room temperature (as all the step henceforth) and washed in PBS 3 times for 3 minutes.
- ***Blocking*** - The eggs were then blocked in 10% BSA for 60 minutes to avoid any unspecific binding and washed in PBS for 5 minutes.
- ***Primary antibodies*** – Blocked eggs were labelled with primary FCRL-3 antibody, dilution 1:200 in 1,5% BSA/PBS for 60 minutes in humidity chamber and washed 3 times for 10 minutes in PBS.
- ***Secondary antibodies*** – For detection of the signal, eggs were incubated with secondary antibody (Alexa Fluor 488, goat anti-rabbit, dilution 1:1000 in 1,5% BSA/PBS) for 60 minutes in dark humidity chamber, followed by 3 washings for 10 minutes.
- ***Mounting on slides*** – The samples were mounted onto microscope slides in a drop of Vectashield medium containing DAPI and sealed with nail polish.
- ***Detection*** - The slides were investigated under inverted fluorescence microscope IX81 and/or under inverted confocal microscope TCS SP2.

4.3.2.2. Immunostaining on fixed complexes of egg with bound sperm

- ***Sperm collection and capacitation*** - Mature male C57Bl/6 mice were sacrificed by cervical dislocation and both their cauda epididymides were placed separately into 200 µl of M2 medium, preheated to 37°C and covered with paraffin oil. The Petri dishes were placed in the incubator (5% CO_2) for 15 minutes to let the sperm swim out of the tissue. This stock was divided by 5µl into new Petri dishes with 100 µl of preheated M2 capacitation media, covered in paraffin oil to approximate final concentration of $5x10^6$ sperm/ml.
- ***Induction of AR*** - For acrosome reaction induction, progesterone was freshly dissolved in DMSO and added to capacitation dishes to final concentration of 10µM for 80 minutes. The final concentration of DMSO in experiment was 1 %, which do not affect the motility or acrosomal integrity (Shi and Roldan, 1995).
- ***Preparation of the eggs and co-incubation*** – The eggs were collected and treated with hyaluronidase and Tyrodes solution (see above). Now zona-free eggs were left at 37°C for 30 minutes to recover, and afterwards co-incubated with sperm for 60 minutes at 37°C and 5% CO_2.

- ***Fixation of the complexes*** – The sperm-egg complexes were washed 3 times in preheated M2 medium to get rid of any loosely attached sperm, and fixed in 3,7% paraformaldehyde for 15 minutes. The proceeding protocol is identical to that of immunostaining on fixed eggs.

4.3.2.3. Immunostaining on live eggs without fixation

- ***Blocking*** – After the removal of *zona pellucida*, eggs were allowed to recover in 10% BSA/PBS for 60 minutes in 37 ℃ to maximally shorten the process, as the eggs tend to degrade or parthenogenetically activate the more and longer they are manipulated with.
- *Co-incubation with sperm* – The eggs were incubated after the blocking step with acrosome reacted sperm (see above) for 40 minutes at 37°C, 5% CO_2. After the incubation, eggs were washed 3 times in preheated M2 medium to get rid of any loosely attached sperm.
- ***Primary antibodies*** – Eggs were checked for any morphological abnormalities or signs of degradation and labelled with primary FCRL-3 antibody, dilution 1:100 in 1,5% BSA/PBS, for 60 minutes in humidity chamber tempered to 37°C. After the staining, eggs were washed 3 times for 10 minutes in PBS.
- ***Secondary antibodies*** – For detection of the signal, eggs were incubated with secondary antibody (Alexa Fluor 488, goat anti-rabbit, dilution 1:1000 in 1,5% BSA/PBS) for 60 minutes in dark humidity chamber tempered to 37°C, followed by 3 washings for 10 minutes.
- ***Mounting on slides*** – The samples were carefully mounted onto microscope slides in a drop of Vectashield medium containing DAPI and sealed with nail polish.
- ***Detection*** - The slides were investigated under inverted fluorescence microscope IX81 in a heated humidity chamber with 5% CO2.

4.3.3. Co-localization of IZUMO1 and FCRL-3

The first step in investigating potential interaction between two proteins on membranes is usually immunfluorescent co-localization of the fluorescent signals. Since the proteins in this case are not both located on the same membrane, but on oolema and attached sperm membrane, the experiment was performed on sperm-egg complex with zona-free eggs. The aim of the study is to investigate interaction between egg and sperm that has potential for

fusion. Therefore the acrosome reaction was induced and only the acrosome reacted sperm attached to egg were analyzed. Progesterone was used for the induction, for it leads to more progressive and faster relocation of IZUMO1, which reaches the whole head staining pattern 80 minutes after the addition of the hormone into capacitation media (Sebkova *et al*., submitted)

The IZUMO1 antibody is designed to work preferably on acetone-fixed smears, which is the type of fixation protocol that is by no means possible for application on eggs, as they almost instantly burst. Together with the fact that the co-localization of FCRL-3 with IZUMO1 must be observed only with the epitopes on the oolema, the sperm-egg complex was not fixed before labelling and the whole experiment was performed on living cells.

Naturally, the secondary antibodies must be labelled with different colours to recognize their overlapping. The signals used in this experiment were green (Alexa Fluor 488, goat anti-rabbit IgG) and red (donkey anti-goat IgG-PE), which were previously proven to work with their belonging primary antibodies. However, there is frequent occurrence of an unspecific and strong background signal, especially in the case of PE secondary antibody. Therefore, the gametes were stained separately, and only after careful and thorough washing steps were they co-incubated for attachment.

Immunostaining on sperm

- ***Sperm collection, capacitation and induction of AR*** – The protocol corresponds to the chapter 4.3.2.3. Mature male C57Bl/6 mice were sacrificed by cervical dislocation and both their cauda epididymides were placed separately into 200 µl of M2 medium, preheated to 37°C and covered with paraffin oil. The Petri dishes were placed in the incubator (5% CO_2) for 15 minutes to let the sperm swim out of the tissue. This stock was divided by 5µl into new Petri dishes with 100 µl of preheated M2 capacitation media, covered in paraffin oil to approximate final concentration of $5x10^6$ sperm/ml.
- ***Induction of AR*** - For acrosome reaction induction, progesterone was freshly dissolved in DMSO and added to capacitation dishes to final concentration of 10µM for 80 minutes. The final concentration of DMSO in experiment was 1 %, which do not affect the motility or acrosomal integrity (Shi and Roldan, 1995).
- ***Primary antibodies*** - Labelling of IZUMO1 protein was carried out by addition of anti-IZUMO1 primary antibody into the dishes 20 minutes after the addition of progesterone

in 1:100 dilution. Blocking of unspecific binding was ensured by adding BSA into mixture at final concentration of 1,5 %.

After 80 minutes from progesterone addition, and also after 60 minutes of labelling the epitope by primary antibody, the sperm was centrifuged for 2 minutes at 3000 rpm. This allowed all the dead and motionless sperm to precipitate at the bottom of the tube. The supernatant with living, moving sperm was collected and centrifuged again for 5 minutes at 3000 rpm. The pellet with sperm was divided into drops of 100 μl of fresh preheated M2 media covered with paraffin oil to final sperm concentration of $5x10^6$ sperm/ml).

- ***Secondary antibodies*** - For signal detection, the secondary antibody Alexa 594 donkey anti-rabbit was added to the dishes at the final dilution of 1:800 for 60 minutes. After incubation, the sperm was centrifuged three times for 3 minutes, each time with the supernatant carefully dispensed of and the pellet dissolved in heated M2 media. This procedure ensured thorough cleaning from any remnants of the secondary antibodies other than those attached. The cleaned and labelled sperm was divided for the last time into fertilization drops of heated M2 media covered with paraffin oil to final sperm concentration $2x10^6$ sperm/ml.

Co-localization assay

The eggs were prepared by the same protocol as in the Immunostaining of the live eggs without fixation (see above). After last thorough washing steps, now FCRL-3 labelled eggs were added into fertilization drops with acrosome-reacted sperm, labelled for IZUMO1 in different colour. After 20 minutes of co-incubation at 37°C, 5% CO_2, the egg-sperm complexes were washed in preheated M2 media to remove all loosely bound sperm and mounted onto microscope slides into preheated Vectashield DAPI mounting medium. The slides were observed under inverted fluorescence microscope IX81 in a heated chamber with 5% CO_2 and analyzed.

4.3.4. Proximity Ligation Assay – Duolink® In Situ

Proximity ligation assay (PLA) is a state-of-art method recognizing closeness of proteins required for their direct interaction. The protein interaction target can be detected and localized with single molecule resolution and quantified in cells and tissues fixed by standard methods. The specificity and sensitivity of immunoassay is achieved by the use of

two secondary oligonucleotide labelled antibodies, which produce the fluorescent signal only when they occur less than 40nm far from each other. This proximity is considered predictive for direct protein interaction. The detection reporter system is based on DNA amplification and detects even transient or weak interactions.

The principle of the assay is not complicated, as it makes use of traditional fluorescence staining. The samples are labelled with target-specific primary antibodies, which must be raised in different species and specifically bind to the proteins of interest. After the washing step, the samples are incubated with species-specific secondary antibodies conjugated with oligonucleotides (PLA probe PLUS and PLA probe MINUS) raised against antibodies from the same animal as the primary ones. They bind to the primary antibodies with the oligonucleotides protruding into the space. In the next, ligation step, the reaction mixture contains two oligonucleotides complementary to the ones conjugated with the secondary antibodies, and an enzyme ligase. If the two secondary antibodies bind in close proximity, the oligonucleotides will hybridize to the two PLA probes and join to form a closed circle by enzymatic ligation. This circle, still attached to both primary antibodies through PLA probes, represents a template for amplification and detection step. The solution for this step consists of nucleotides and fluorescently labelled oligonucleotides with polymerase. One arm of DNA strand on of the PLA probes represents a primer for the enzyme, which starts the rolling-circle amplification using the ligated circle as a template and nucleotides as building blocks. The fluorescently labelled oligonucleotides in the mixture finally hybridize with the concatemeric product of the rolling-circle amplification and produce a distinct fluorescence spot which can be view by fluorescence microscopy.

Duolink® In Situ was selected for our research as it clearly identifies very close vicinity of two proteins, which could prove either the interaction of IZUMO1 and FCRL-3, or at least their co-localization in the fusion-complex and possible role in the fusion machinery.

4.3.4.1. Positive and negative control for PLA

For positive control of the experiment we decided to use the results of Vesterlund et al. (2011), who researched the co-localization of neural cell adhesion molecule (NCAM) and fibroblast growth factor receptor 2 (FGFR2) and pioneered a single oocyte adaptation of the proximity ligation assay. The results of the study were positive – molecules were found to interact thanks to the PLA method - so the same primary antiobodies as in Vesterlund´s study were used to optimize and verify the method in our laboratory.

To examine the PLA probe background signals, the technical negative control was used by omitting both primary antibodies.

4.3.4.2. Detailed procedure of Duolink® In Situ positive control (by User manual)

Because of high sensitivity of the method, all the steps were performed in flow box with sterile filtered reagents. Eggs were collected and treated to get zona-free cells (see above), fixed in 3,7% paraformaldehyde for 15 minutes and after careful washing (3 times for 10 minutes in PBS to get rid of possible fixation precipitates) placed into blocking solution (10% BSA/PBS) for 60 minutes in humidity chamber.

- ***Primary antibodies*** – Primary antibodies against NCAM and FGFR2 were diluted in the same diluent (1,5% BSA/PBS) to 1:400 and 1:300 respectively. The blocked eggs were washed in PBS and transferred into wells with primary antibodies solution, where they incubated in humidity chamber for 90 minutes at room temperature.
- ***PLA probes*** – Both of the PLA probes were mixed with antibody diluent (1,5% BSA/PBS) to 1:5 concentration. The mixture was allowed to sit for 20 minutes at room temperature. The eggs were thoroughly washed from primary antibodies with PBS (3 times 5 minutes) on shaker, added to the PLA probes solution and incubated in a humidity chamber for 60 minutes at 37°C.
- ***Ligation*** – The ligation stock solution was thawed, vortexed and diluted 1:5 in high purity water. The eggs were washed 2 times for 5 minutes in Wash Buffer A under gentle agitation to get rid of all unattached PLA probes. Meanwhile, the ligase enzyme was removed from the freezer using a freezing block and added into the diluted ligation stock solution at a 1:40 dilution. Before the addition of eggs, the ligase-ligation mixture was carefully vortexed and the wells with eggs were incubated in a humidity chamber for 30 minutes at 37°C.
- ***Amplification*** – This represents the detection part of the method with light-sensitive reagents, therefore all the steps were performed in a dark room. The amplification stock was diluted 1:5 in high purity water and vortexed thoroughly while the eggs were washed in Wash Buffer A 2 times for 2 minutes. The polymerase enzyme was removed from the freezer using a freezing block and added to the diluted amplification solution at

a 1:80. The mixture was vortexed again and the washed eggs were added to be incubated in a humidity chamber for 100 minutes at 37°C.

- ***Final washing*** – The eggs were transferred into Wash Buffer B and thoroughly washed on 2 times for 10 minutes the shaker placed in dark. For final washing, 0,01xWash Buffer B was prepared by diluting the solution 1:100 in high purity water. The eggs were washed in this diluted buffer for 1 minute.
- ***Mounting on slides*** – The eggs were mounted onto the microscope slides into Duolink In Situ Mounting Medium with DAPI and the edges were sealed with nail polish. The slides were stored in a freezer before fluorescence microscope imaging.

4.3.4.3. The course of actions in optimizing the method

As the results of negative control for the PLA assay rendered positive signal, we resorted to setting up a side by side experiments omitting different steps from the process, like PLA probes, ligation or amplification step. The effectiveness of the PLA probes was checked by using the same ones, but from the different lot, kindly provided by Ms. Toralova from Institute of Animal Physiology and Genetics AS CR, v.v.i.

5. Results

5.1. Distribution of FCRL3 in the mouse egg

5.1.1. FCRL3 immunostaining on paraformaldehyde fixed eggs

The zona-free eggs were fixed, labelled with anti-FCRL3 primary antibody and its corresponding secondary antibody and observed under fluorescent microscope. The signal was found to be strong throughout the egg (Fig. 1). The exact distribution and possible plasmatic membrane localization is not evident. Therefore the fixed eggs were inspected under confocal microscope with sections acquired stepwise by 2 µm throughout the egg. The data were put together to form a short lapsed 3dimensional video (see Attachment, eclosed CD) with a distinct layout of the fluorescent signals.

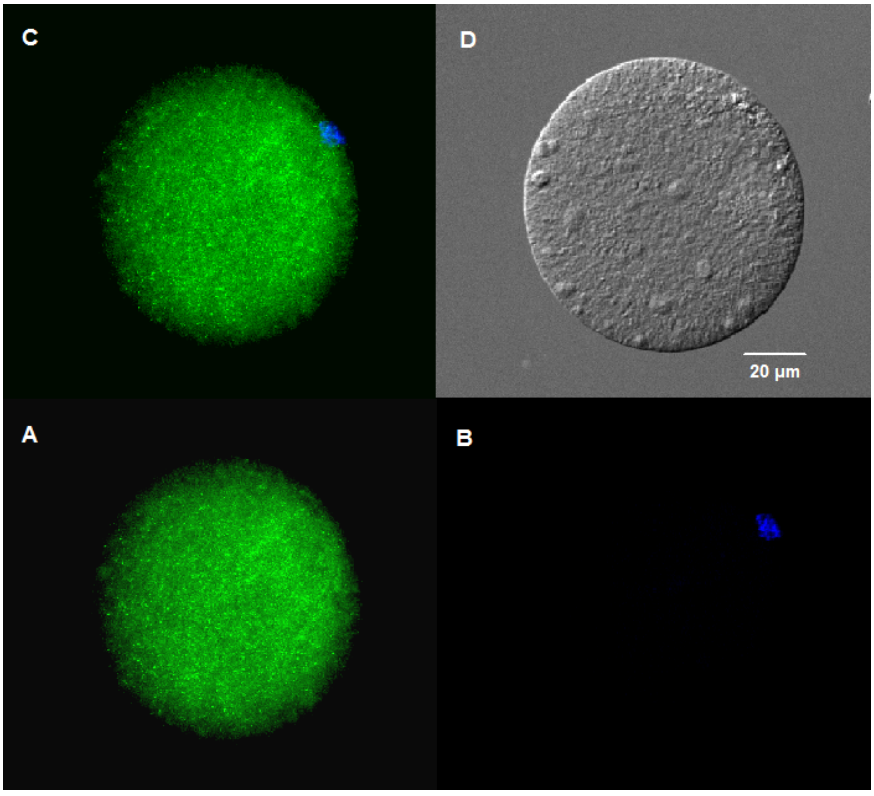

Figure 1: FCRL3 immunostaining on fixed eggs. Zona-free paraformaldehyde fixed egg (bright field DIC) (D), stained against FCRL3 (green) (A) and against chromatin with DAPI (blue) (B), merged (C). The recorded staining pattern was visible in all observed eggs (n>10).

5.1.2. FCRL3 immunostaining on fixed sperm-egg complexes

To assess the possible change in FCRL3 staining pattern after the attachment of sperm to egg plasmatic membrane, sperm-egg complexes were fixed and labelled for FCRL3 (Fig. 2). There appeared no detectable alteration in the signal layout compared to the eggs labelled without any contact with sperm. The localization of FCRL3 is not restricted to micriovilli-rich area, as the staining pattern does not change over the mitotic spindle.

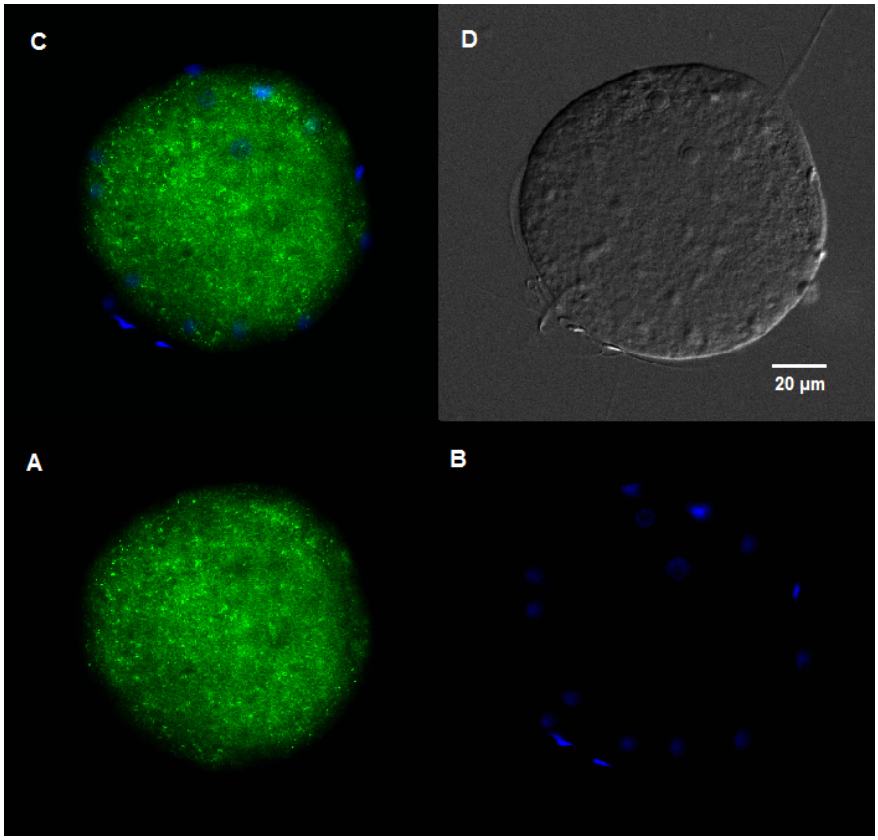

Figure 2: FCRL3 immunostaining on fixed sperm-egg complex. Zona-free eggs were incubated with sperm for 60 minutes, subsequently fixed with paraformaldehyde and labelled for FCRL3. Bright field DIC (D), fluorescent labelling against FCRL3 (green) (A) and against chromatin with DAPI (blue) (B), merged (C). The recorded staining pattern was visible in all observed eggs (n>10).

5.1.3. FCRL3 immunostaining on unfixed cells

The FCRL3 antibody enters the cell via permeabilized fixed membrane and thus the possible signal on the membrane is indistinguishable. Therefore the immunolabelling was carried out

on living, unfixed egg, where the penetration of the antibody is not expected. The eggs were co-incubated with sperm prior to labelling to enhance the stability of the cells during demanding immunostaining process. The staining showed a distinct signal on the surface of the egg, greatly reduced in numbers compared to the immunostaining of fixed eggs (Fig. 3).

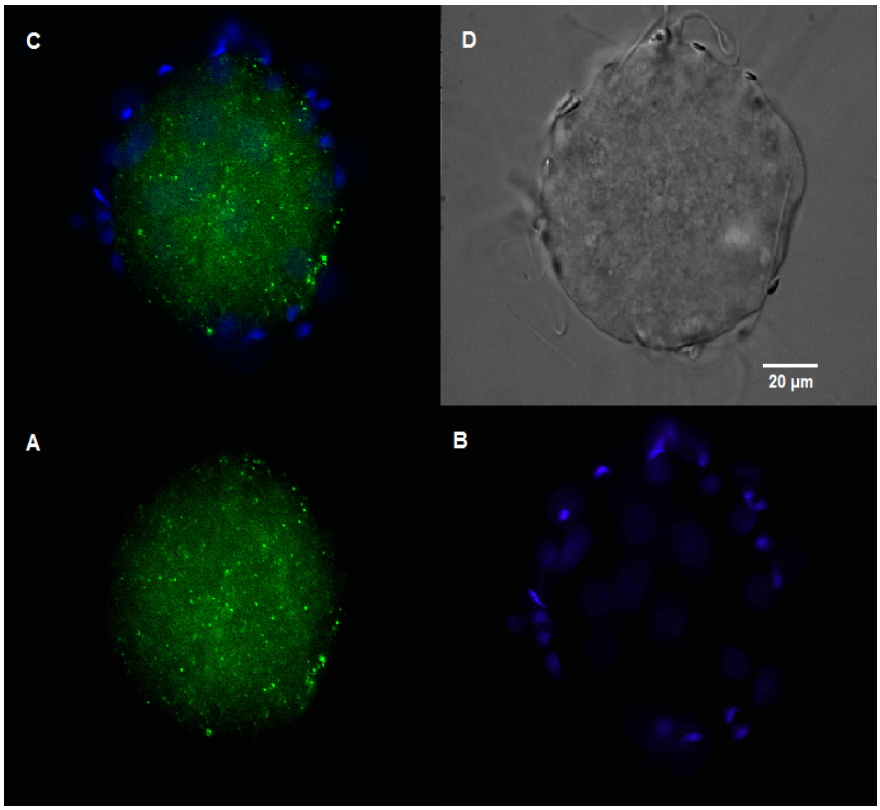

Figure 3: FCRL3 immunostaining on unfixed live eggs. Zona-free egg coincubated with sperm and subsequently stained with FCRL3 antibody without fixation step. Bright field DIC (D), staining against FCRL3 (green) (A) and against chromatin with DAPI (blue) (B), merged (C).

5.2. Co-localization assay

The possible proximity of the IZUMO1 on sperm and FCRL3 on egg at the site of gametes attachment was primarily investigated by co-localization of multicolour immunofluorescence. The sperm-egg complexes were labelled without fixation and observed by fluorescence microscopy. The sperm is attached by mid-lateral portion of the head. The IZUMO1 signal demonstrates the whole head distribution indicating the sperm is acrosome reacted, and overlaps the signal of FCRL3, indicating the co-presence of these proteins at the site of the attachment.

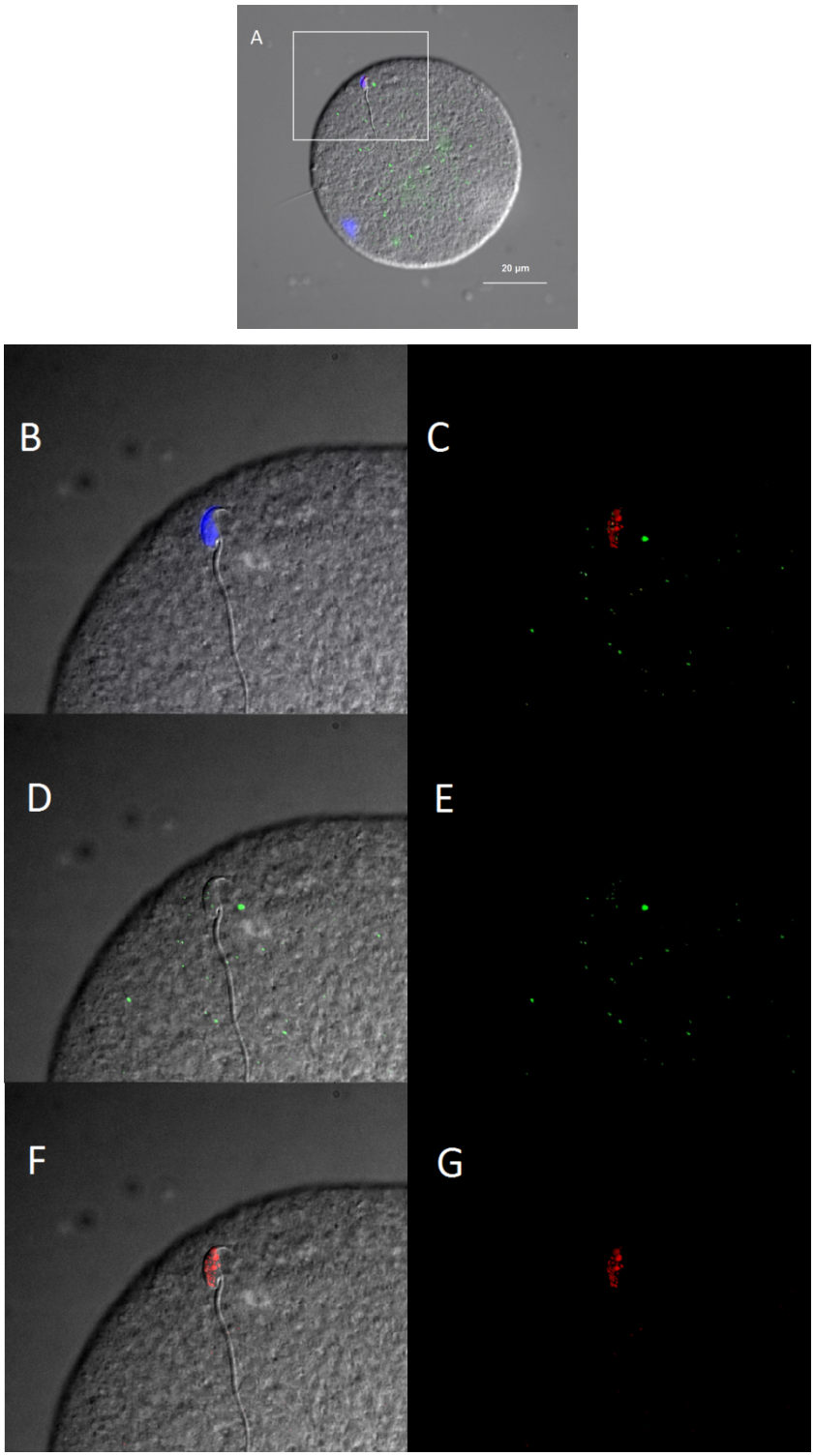

Figure 4: Co-localization of IZUMO1 and FCRL3 on unfixed eggs with attached sperm. FCRL3 labelled green, IZUMO1 labelled red, chromatin by DAPI blue. (A) Egg with attached sperm and all three signals merged. The rectangle shows the site of following close-ups. (B) Bright field merged with DAPI, (C) FCRL3 and IZUMO1 co-localization, green and red merged, (D) bright field merged with FCRL3 green signal, (E) FCRL3 green signal, (F) Bright field merged with IZUMO1 red, (G) IZUMO1 red signal.

5.3. Proximity Ligation Assay

5.3.1. Technical negative control without primary antibodies

While optimizing the method in our laboratory, we chose the technical negative control to give us an idea about background signal that needs to be counted with. This experiment consists of the same experimental protocol as the usual one, only without any primary antibodies to bind the PLA probes. For further assessing, we conducted negative control without primary antibodies and either of the PLA probes, to assess their possible effect on the outcome signal.

The results of negative control with our PLA probes are shown in the Figure 5. A distinctive signal can be seen in all three experiments (with both PLA probes, only with MINUS, only with PLUS) as the singular red dots.

To find out whether the problem laid in our PLA probes, we borrowed a different ones from the IAPG A.S. The results of negative control with these secondary antibodies are shown in the Figure 6. The singular signal can also be seen in all three design experiments, suggesting the source of non-specificity in our experiment does not lay in the PLA probes used.

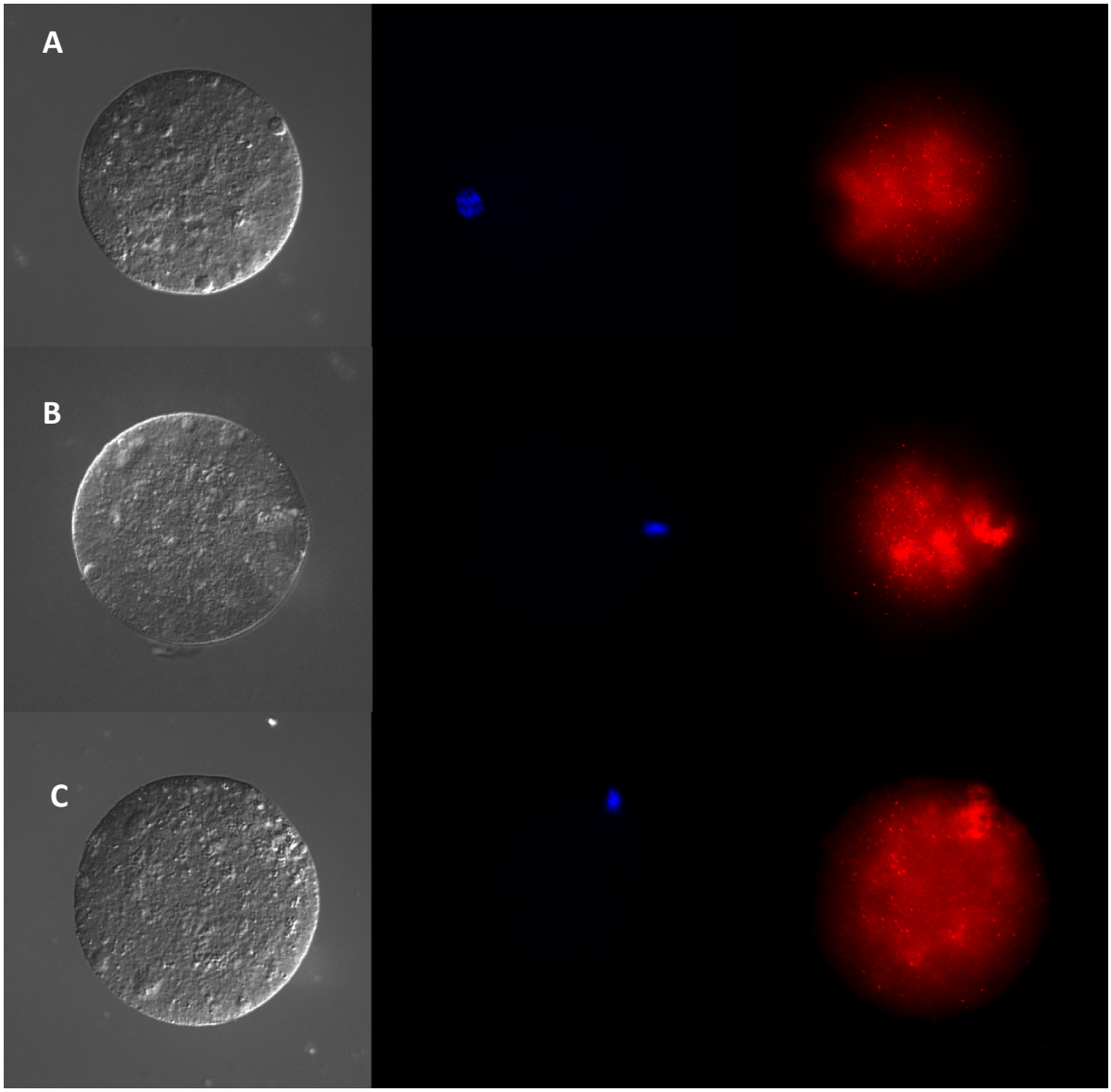

Figure 5: Proximity ligation assay technical negative control without any primary antibodies. The signal of the assay is shown in red, the immunostaining of chromatin with DAPI shown in blue. (A) The negative control with both PLA probes, (B) the negative control with only MINUS PLA probe, (C) the negative control with only PLUS PLA probe. The distinctive red dots shows the same signal appearance as expected in positive result of full experiment.

5.3.2. Side by side experiments

To evaluate the possibility of solutions used in the Duolink protocol as the cause of unspecific signal in negative control, side by side experiment has been set up with different steps omitted from the original experimental design. The exact lay-out of the experiment is shown in Table 1. The results (Figure 6) show that the unspecific signals encountered in negative control are caused by both enzymatic steps in the course of the experiment (ligation and/or polymerization), as the experiment with nothing but any of these steps still provides distinct fluorescent spots on the egg.

No. of experiment	Fixation	Blocking	Ligation	Polymerization
1	3,7% PFA	10% BSA	Ligation	Polymerization
2	3,7% PFA	10% BSA	Ligation	
3	3,7% PFA	10% BSA		Polymerization

Table 1: The experimental layout of side by side experiment to determine the effect of each individual detection step in the PLA design.

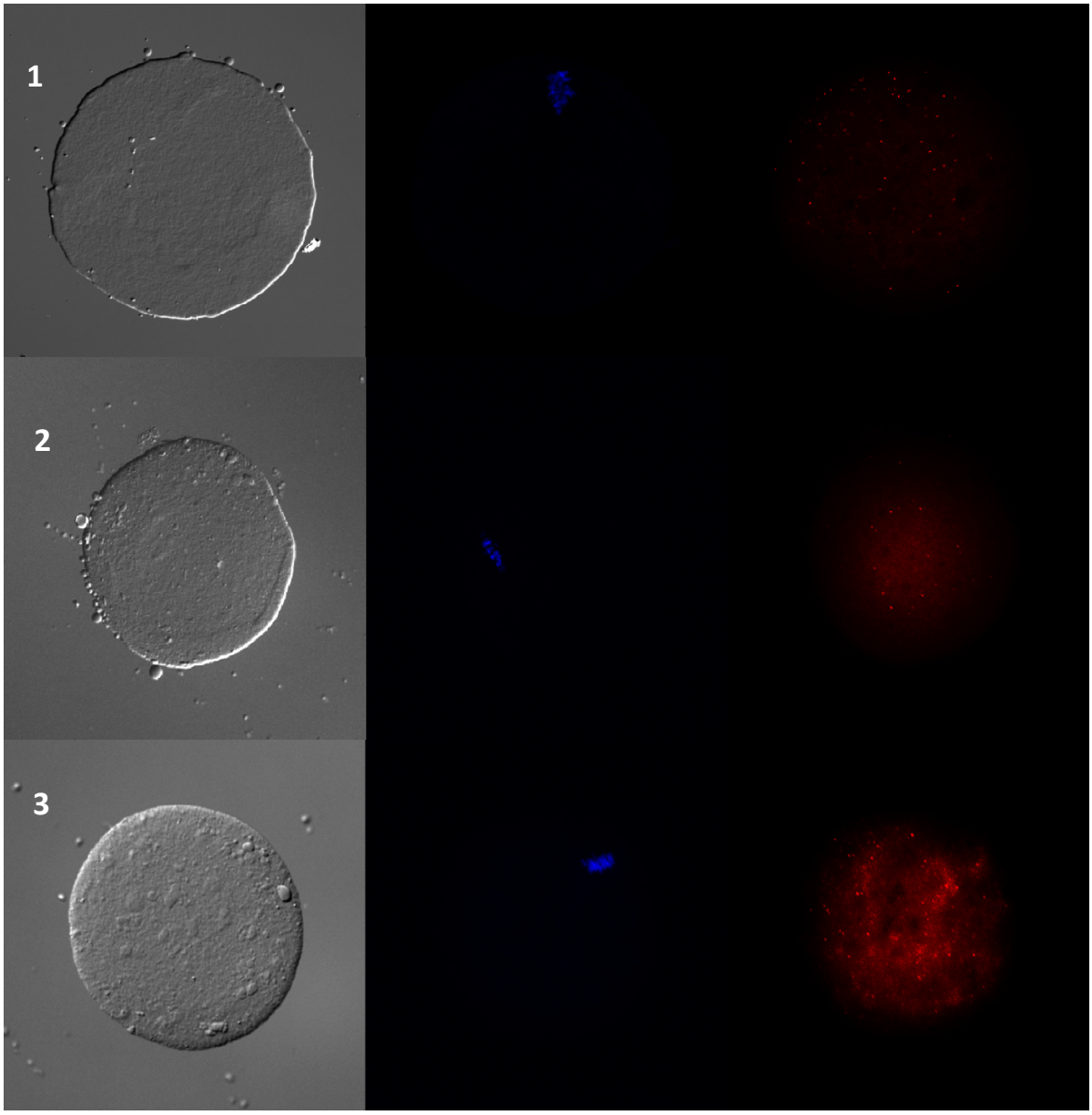

Figure 6: The results of side by side experiments to evaluate the solutions used as a possible cause of unspecific signal. Signal of the assay is labelled in red, the staining of chromatin by DAPI blue. A detailed layout of each experiment is shown in Table 1. (1) Test without any primary antibodies or PLA probes, (2) test without primary antibodies, PLA probes and polymerization step, (3) test without primary antibodies, PLA probes and amplification step. All of the designs show distinctive red signal, suggesting the solutions, probably those of enzymatic steps, cause the unspecificity.

6. Discussion

Fusion is a critical constituent of sexual reproduction, consisting of coordinated steps culminating in the merger of plasmatic membranes. This cytoplasmic union is achieved through gamete interactions, specifically cell adhesion and subsequent membrane fusion of the gamete plasma membranes. All its molecular components or exact mechanism in mouse are not yet known, but some players have been found and an overall concept is emerging. The only up to date known essential or nearly essential players in gamete membrane interaction are IZUMO1 on sperm (Inoue et al., 2005) and CD9 on egg (Miyado *et al*., 2000; Le Naour *et al.*,2000; Kaji *et al*.; 2000)

The main advantage of knowing at least some factors playing role in an interaction is the fact that we can use this molecule to look for its binding partners, associated molecules or signalling pathways. Since IZUMO1 contains an immunoglobulin domain (Ig), which is known for its interaction with various molecules, it is predicted that it binds to some receptor on the surface of the egg membrane. However, this molecule remains unknown. IZUMO1, often described as a primary fusogen of the sperm, lacks features associated with fusogenic molecule and most likely plays a role in gamete binding. Ig domain is known for for binding with ligands on other cells (in trans) as well as for interactions with ligands expressed on the same cell (in *trans*), therefore IZUMO1 can be a binding partner as well as a membrane-associated protein of the *bona fide* fusogen.

The hypothesis of IZUMO1 interlinking the space between gametes by interaction with an unknown oolemal factor opens the search for such a molecule. Known connection between Ig and Fc domains led to a pinpointing a candidate-binding partner for IZUMO1, Fc receptor-like 3, by Professor Harry Moore from Sheffield University. The presence of FCRL-3 on mouse egg and its potential role in gamete interaction was investigated in this study by immunofluorescence staining, co-localization of the signal with that of IZUMO1 and proximity ligation assay, a rather new method in cellular biology.

In presented study, at first the FCRL-3 antibody was optimized for mouse superovulated eggs. The protein contains a transmembrane domain and is predicted to be an integral membrane inhabitant of all cellular membranes, specifically plasma membrane. The antibody was raised against extracellular part of the protein, so its signal was anticipated to be found on the surface of the cell. Our data from imaging immunofluorescently stained fixed eggs with permeable membranes showed the signal spread unevenly across the

cytoplasm. The experiment was repeated with unfixed, living eggs and short incubation time with primary antibody, where its intake into the cell is unlikely. This setting gave distinct signal on the surface of the cell, proving the presence of antigen on the plasma membrane of egg and supporting its potential role as plasmatic receptor.

The intracellular presence of the antigen was visualized by 3D imaging – collecting confocal section images stepwise through the cell from one end of an egg (plasma membrane), moving towards the other end by 2μm steps. These images were then put together to form spatial image that can be viewed from different angles, forming a short lapsed video (see Attachment, enclosed CD). This showed an uneven intracellular distribution of the signal forming clusters close to the centre of the cell. This order of dispersion can be explained by the presence of the antigen in intracellular membrane structures delivering membrane factors to the oolema, and by potential presence in a secretory pathway. Also binding of the antibody to some intracellular molecule structurally close to FCRL3 cannot be ruled out.

The expression of membrane factors playing parts in cell-cell interaction often changes during the course of their cooperation. For this reason, the possible change in FCRL3 signal distribution was assessed on eggs after the sperm attachment. This experiment did not show any visible change in signal intensity or distribution. However, the presence of antigen on the plasmatic membrane was confirmed and so the potential binding with IZUMO1 on sperm could be investigated.

The research demanded good experimental design to assess the hypothesis. The ongoing hunt for molecules responsible for fusion uses a battery of well established methods, including use of anti-gamete monoclonal antibodies subsequently tested in *in vitro* sperm-egg binding assays and in *in vitro* fertilization function-blocking experiments. This way, IZUMO1 protein was identified at first. This study´s aim was to look at potential binding partners, hence the sperm-egg binding assay had to be considered as a method.

When designing the experiment, we found the possible interpretation of these assays problematic due to lack of physiologically meaningful *in vitro* sperm-oocyte binding assay (Talbot *et al*., 2003). Sperm binding prior to fusion is a stepwise process starting with loose attachment and progressing to stronger adhesion potentially leading to fusion. Even sperm unable of membrane merging, as acrosome-intact sperm, still binds to oolema of zona-free eggs, however transiently. Most of the sperm attached in a typical binding assay are unable to process to fusion, it is difficult to distinguish between sperm that is merely attached and sperm that is truly bound, and interpretation might be difficult due to various methods of *zona pellucida* removal, which may not be fully removed and sperm can bound to its

remnants (Yamagate *et al.*, 2002; Evans *et al.*, 1997). These problems partly explain why so many factors considered important for fusion have been proven dispensable by recent genetic disruption experiments (i.e. He *et al.*, 2003; Inoue *et al.*, 2012). Therefore we decided against use of binding assay in assessing FCRL-3 role in gamete interaction.

The suitable method for primal determination whether it is possible for these two molecules to interact was set up to be immunofluorescent co-localization assay. It is frequently used to examine possible protein-protein direct interactions, as they need to be fairly close to each other for the fluorescent signals to overlap. Methodically it has its limits, especially in interpretation, as the observed overlapping of the signals from two separate emission wavelengths can refer to mere co-occurance, presence of two fluorophores in the same pixel and represents a bias caused by visual interpretation. As the sperm seem to be attached by a relatively large portion of the head and it is not possible to distinguish signal from individual molecules, we can say that the site FCRL3 localizes at the site of the attachment. This certainly does not signify the interaction of IZUMO1 and FCRL3, but rather the possibility and chance of such function.

Thus the co-presence of FCRL-3 and IZUMO1 at the site of attachment was confirmed. Even though this does not constitute the proof of their interaction, the hypothesis about their receptor cooperation cannot be excluded.

The closeness required for two objects to interact can be detected by proximity ligation assay (PLA) Duolink® In Situ. In this method (two specific primary antibodies, in our case against-FCRL3 and against-IZUMO1, are coincubated onto the sample. If they attach closer than 40nm far from each other, they are detected by binding of PLA probes, secondary antibodies with oligonucleotide chains and their subsequent hybridization with fluorescent labelled nucleotides forming a detectable product by amplification (Fredriksson *et al.*, 2002). The method is very sensitive and capable of visualizing an individual interaction as a distinct spot.

The PLA experiment was conducted with technical negative control consisting of omitting both of the primary antibodies, which was supposed to give an idea of background signal. Surprisingly, the images of eggs from the negative control showed a collection of distinct spots undistinguishable from the copybook positive results of the method. This prevents the analysis of the method, as the background cannot be differentiated from a possible positive signal, therefore we had to recourse at first for troubleshooting and optimizing the negative control.

According to suggested solutions in troubleshooting from the manufacturer, all the solutions were sterile filtered or new ones were used, and washing steps were prolonged to ensure no dust, salt or fixation precipitates remain in the samples, but the results were still in spotty manner across the surface of the egg. New PLA probes were checked and side-by-side experiments skipping different steps in the protocol were set up to find out which step renders such an unspecific and unparalleled signal in the negative control.

As the signal remained even when PLA probes and ligation step were omitted, it was decided that the lot of solutions used for amplification of the signal were contaminated with an unknown substance adhering to random places in the egg and giving signal undistinguishable from the positive signal. This of course prevented the experiments from any analysis, as the difference between present positive signal and background could not be told apart. Thus the close proximity of our proteins of interest could not be determined by this method.

Preliminary results of this analysis of FCRL-3 distribution on the surface of egg membrane show that this molecule might play a role in the adhesion or fusion of the gametes as the IZUMO1 binding partner, or as an associated protein of other molecules in *cis*-interaction. The results could not be broadened by the results of PLA assay due to unexpected difficulties.

A key test in assessing the role of any factor today is its genetic deletion in knock-out system. It would be intriguing to see whether gene deletion in *FcRL-3* gene would lead to any effect on reproductive function. A failure to produce any offspring, a phenotype demonstrating an essential role of the factor in question is a rare outcome, which only *Izumo1*$^{-/-}$ mice showed up to date. If the females exhibit subfertile phenotype, it might indicate that the molecule works in redundant ways with complementary molecules. This outcome might be considered as less informative, but the case of CD81 and CD9 shows that even moderately subfertile phenotype can provide significant insight. CD9-null females have severely reduced fertility (Miyado *et al*., 2000; Le Naour *et al.*, 2000; Kaji *et al*.; 2000), whereas CD81-null mice show only mild decrease in reproduction (Kaji *et al*., 2002). Even though CD81 was at first considered a redundant and not distinctively important factor in reproduction, the phenotype of double knock out *Cd9*$^{-/-}$*/Cd81*$^{-/-}$ was shown to be completely infertile and proved the importance assessing subtle changes in reproductive phenotype for understanding fully the complex machinery of the gametes. The concentration on only binary assessment of fertility (like any pups versus no pups) could result in missing

on certain molecules contributing to reproductive success by less robust systems (Evans, 2012).

Owing to the fact that even knock-out experiments have their limits in interpretation, methods like co-localization and proximity ligation assay may prove to provide significant insight into molecular interaction leading to the formation of zygote. The working hypothesis in the field of gamete interaction is, that various proteins in the adhesion/fusion machinery may have various different roles than just *trans*-interactions and adhesion, but also *cis*-interaction regulating membrane morphology, functionality and cellular signalling. As the FCRL3 is known to be connected to a tyrosine signal transduction path, if ever proven to be the binding receptors, it might trigger the fusion by intracellular signalling in a yet unknown way. FCRL3 does not possess any strictly speaking fusogenic domain and is therefore regarded as a possible attachment/binding receptor, orchestrating the cellular events by this very intracellular signalling.

The answers for the question whether IZUMO1 binds to FCRL3 are far from complete and a lot of work is yet to be done on the matter to determine the actual role of the FCRL3 molecule in gamete interaction. However, the data do not disprove of hypothesis that it might represent the long sought-after partner of IZUMO1 and may play a role in fusogenic machinery on the egg surface.

7. Conclusion

Fc receptor-like 3 molecule (FCRL3) has been found intracellularly and on the surface of mouse egg by immunstaining. The presence inside the cell can be explained by localization on various cell membranes, detectable by permeabilization of fixed eggs, while the extracellular signal implies that the molecule can play a role as a ligand or receptor in gamete interaction. The distribution of the signal has not shown any pattern over the oolemal surface, suggesting the localization of FCRL3 is not restricted to the microvilli-rich region of the egg. The attachment of sperm seems to have no effect on the evenly distribution of the molecule over the surface.

The possible co-presence of FCRL3 and IZUMO1 as its potential binding partner on the sperm was assessed by co-localization of immunofluorescent signal. FCRL3 is detectable at the site of sperm attachment and the signal overlaps with that of IZUMO1 on sperm. This finding suggests that these molecules might directly or indirectly interact or play other role in fusion machinery as they are both find at the site of fusion.

A very close proximity of FCRL3 and IZUMO1, which would strongly indicate their direct interaction, could not be assessed by Proximity ligation assay. This method could not be analyzed due to encountered problems with unspecific signals in negative control and thus an impossible detection of the positive signal in experiment.

This study presents preliminary results in research of gamete binding receptors. By our findings, FCRL3 cannot be ruled out as a potential binding partner of IZUMO1, as it localizes at the site of sperm attachment and fusion in mouse eggs.

8. List of references

Aguilar, P. S., M. K. Baylies, A. Fleissner, L. Helming, N. Inoue, B. Podbilewicz, H. Wang and M. Wong (2013). *Genetic basis of cell-cell fusion mechanisms*. Trends Genet 29(7): 427-437.

Alfieri, J. A., A. D. Martin, J. Takeda, G. Kondoh, D. G. Myles and P. Primakoff (2003). *Infertility in female mice with an oocyte-specific knockout of GPI-anchored proteins*. J Cell Sci 116(Pt 11): 2149-2155.

Ashida, E. R. and V. L. Scofield (1987). *Lymphocyte major histocompatibility complex-encoded class II structures may act as sperm receptors*. Proc Natl Acad Sci U S A 84(10): 3395-3399.

Chan, D. C. and Kim, P. S. (1998). "HIV entry and its inhibition". *Cell* 93 (5): 681–4

Chen, A., E. Leikina, K. Melikov, B. Podbilewicz, M. M. Kozlov and L. V. Chernomordik (2008). *Fusion-pore expansion during syncytium formation is restricted by an actin network*. J Cell Sci 121(Pt 21): 3619-3628.

Chen, M. S., K. S. Tung, S. A. Coonrod, Y. Takahashi, D. Bigler, A. Chang, Y. Yamashita, P. W. Kincade, J. C. Herr and J. M. White (1999). *Role of the integrin-associated protein CD9 in binding between sperm ADAM 2 and the egg integrin alpha6beta1: implications for murine fertilization*. Proc Natl Acad Sci U S A 96(21): 11830-11835.

Claas, C., C. S. Stipp and M. E. Hemler (2001). *Evaluation of prototype transmembrane 4 superfamily protein complexes and their relation to lipid rafts*. J Biol Chem 276(11): 7974-7984.

Coonrod, S. A., S. Naaby-Hansen, J. Shetty, H. Shibahara, M. Chen, J. M. White and J. C. Herr (1999). *Treatment of mouse oocytes with PI-PLC releases 70-kDa (pI 5) and 35-*

to 45-kDa (pI 5.5) protein clusters from the egg surface and inhibits sperm-oolemma binding and fusion. Dev Biol 207(2): 334-349.

Dimitrov, D. S. (2004). *Virus entry: molecular mechanisms and biomedical applications*. Nat Rev Microbiol 2(2): 109-122.

Dupressoir, A., G. Marceau, C. Vernochet, L. Bénit, C. Kanellopoulos, V. Sapin and T. Heidmann (2005). *Syncytin-A and syncytin-B, two fusogenic placenta-specific murine envelope genes of retroviral origin conserved in Muridae*. Proc Natl Acad Sci U S A 102(3): 725-730.

Ebensperger, C. and Barros, C. *(*1984*). Changes at the hamster oocyte surface from the germinal vesicle stage to ovulation.* Gamete Res. 9*,* 387-*397.*

Ellerman, D. A., C. Ha, P. Primakoff, D. G. Myles and G. S. Dveksler (2003). *Direct binding of the ligand PSG17 to CD9 requires a CD9 site essential for sperm-egg fusion*. Mol Biol Cell 14(12): 5098-5103.

Ellerman, D. A., J. Pei, S. Gupta, W. J. Snell, D. Myles and P. Primakoff (2009). *Izumo is part of a multiprotein family whose members form large complexes on mammalian sperm*. Mol Reprod Dev 76(12): 1188-1199.

Evans, J. P. (2012). *Sperm-egg interaction*. Annu Rev Physiol 74: 477-502.

Evans, J. P., G. S. Kopf and R. M. Schultz (1997). *Characterization of the binding of recombinant mouse sperm fertilin beta subunit to mouse eggs: evidence for adhesive activity via an egg beta1 integrin-mediated interaction*. Dev Biol 187(1): 79-93.

Farquhar, M. J., Harris, H. J. and J. A. McKeating (2011) *Hepatitis C virus entry and the tetraspanin CD81*. Biochem Soc Trans 39: 532–536.

Fredriksson, S., M. Gullberg, J. Jarvius, C. Olsson, K. Pietras, S. M. Gústafsdóttir, A. Ostman and U. Landegren (2002). *Protein detection using proximity-dependent DNA ligation assays*. Nat Biotechnol 20(5): 473-477.

Friedman, E. A. and Rutherford, J. W (1956). *Pregnancy and Lupus Erythematosus*. Obstretrics and Gynecology 8(5): 601-610

Glazar, A. I. and J. P. Evans (2009). *Immunoglobulin superfamily member IgSF8 (EWI-2) and CD9 in fertilisation: evidence of distinct functions for CD9 and a CD9-associated protein in mammalian sperm-egg interaction*. Reprod Fertil Dev 21(2): 293-303.

Harrison, S. C. (2008). *Viral membrane fusion*. Nature Structural and Molecular Biology, 15(7): 690-698

He, Z. Y., C. Brakebusch, R. Fässler, J. A. Kreidberg, P. Primakoff and D. G. Myles (2003). *None of the integrins known to be present on the mouse egg or to be ADAM receptors are essential for sperm-egg binding and fusion*. Dev Biol 254(2): 226-237.

Herrero, M. B., A. Mandal, L. C. Digilio, S. A. Coonrod, B. Maier and J. C. Herr (2005). *Mouse SLLP1, a sperm lysozyme-like protein involved in sperm-egg binding and fertilization*. Dev Biol 284(1): 126-142.

Horváth, G., V. Serru, D. Clay, M. Billard, C. Boucheix and E. Rubinstein (1998). *CD19 is linked to the integrin-associated tetraspans CD9, CD81, and CD82*. J Biol Chem 273(46): 30537-30543.

Inoue, N., M. Ikawa, A. Isotani and M. Okabe (2005). *The immunoglobulin superfamily protein Izumo is required for sperm to fuse with eggs*. Nature 434(7030): 234-238.

Inoue, N., M. Ikawa and M. Okabe (2011). *The mechanism of sperm-egg interaction and the involvement of IZUMO1 in fusion*. Asian J Androl 13(1): 81-87.

Inoue, N., T. Nishikawa, M. Ikawa and M. Okabe (2012). *Tetraspanin-interacting protein IGSF8 is dispensable for mouse fertility*. Fertil Steril 98(2): 465-470.

Jahn, R., T. Lang and T. C. Südhof (2003). *Membrane fusion*. Cell 112(4): 519-533.

Johnson, M. H., D. Eager, A. Muggleton-Harris and H. M. Grave (1975). *Mosaicism in organisation concanavalin A receptors on surface membrane of mouse egg*. Nature 257(5524): 321-322.

Jégou, A., A. Ziyyat, V. Barraud-Lange, E. Perez, J. P. Wolf, F. Pincet and C. Gourier (2011). *CD9 tetraspanin generates fusion competent sites on the egg membrane for mammalian fertilization*. Proc Natl Acad Sci U S A 108(27): 10946-10951

Kaji, K., S. Oda, S. Miyazaki and A. Kudo (2002). *Infertility of CD9-deficient mouse eggs is reversed by mouse CD9, human CD9, or mouse CD81; polyadenylated mRNA injection developed for molecular analysis of sperm-egg fusion*. Dev Biol 247(2): 327-334.

Kaji, K., S. Oda, T. Shikano, T. Ohnuki, Y. Uematsu, J. Sakagami, N. Tada, S. Miyazaki and A. Kudo (2000). *The gamete fusion process is defective in eggs of Cd9-deficient mice*. Nat Genet 24(3): 279-282.

Kochi, Y., R. Yamada, A. Suzuki, J. B. Harley, S. Shirasawa, T. Sawada, S. C. Bae, S. Tokuhiro, X. Chang, A. Sekine, A. Takahashi, T. Tsunoda, Y. Ohnishi, K. M. Kaufman, C. P. Kang, C. Kang, S. Otsubo, W. Yumura, A. Mimori, T. Koike, Y. Nakamura, T. Sasazuki and K. Yamamoto (2005). *A functional variant in FCRL3, encoding Fc receptor-like 3, is associated with rheumatoid arthritis and several autoimmunities*. Nat Genet 37(5): 478-485.

Le Naour, F., M. André, C. Boucheix and E. Rubinstein (2006). *Membrane microdomains and proteomics: lessons from tetraspanin microdomains and comparison with lipid rafts*. Proteomics 6(24): 6447-6454.

Le Naour, F., E. Rubinstein, C. Jasmin, M. Prenant and C. Boucheix (2000). *Severely reduced female fertility in CD9-deficient mice*. Science 287(5451): 319-321.

Lefèvre, B., J. P. Wolf and A.. Ziyyat (2010). *Sperm-egg interaction: is there a link between tetraspanin(s) and GPI-anchored protein(s)?*. Bioessays 32(2): 143-152.

Li, F. J., D. M. Schreeder, R. Li, J. Wu and R. S. Davis (2013). *FCRL3 promotes TLR9-induced B-cell activation and suppresses plasma cell differentiation*. Eur J Immunol.

Miyado, K., G. Yamada, S. Yamada, H. Hasuwa, Y. Nakamura, F. Ryu, K. Suzuki, K. Kosai, K. Inoue, A. Ogura, M. Okabe and E. Mekada (2000). *Requirement of CD9 on the egg plasma membrane for fertilization*. Science 287(5451): 321-324.

Miyado, K., K. Yoshida, K. Yamagata, K. Sakakibara, M. Okabe, X. Wang, K. Miyamoto, H. Akutsu, T. Kondo, Y. Takahashi, T. Ban, C. Ito, K. Toshimori, A. Nakamura, M. Ito, M. Miyado, E. Mekada and A. Umezawa (2008). *The fusing ability of sperm is bestowed by CD9-containing vesicles released from eggs in mice.* Proc Natl Acad Sci U S A 105(35): 12921-12926.

Ohnami, N., A. Nakamura, M. Miyado, M. Sato, N. Kawano, K. Yoshida, Y. Harada, Y. Takezawa, S. Kanai, C. Ono, Y. Takahashi, K. Kimura, T. Shida, K. Miyado and A. Umezawa (2012). *CD81 and CD9 work independently as extracellular components upon fusion of sperm and oocyte*. Biol Open 1(7): 640-647.

Okabe, M., M. Yagasaki, H. Oda, S. Matzno, Y. Kohama and T. Mimura (1988). *Effect of a monoclonal anti-mouse sperm antibody (OBF13) on the interaction of mouse sperm with zona-free mouse and hamster eggs*. J Reprod Immunol 13(3): 211-219.

Oren-Suisse, M. and Podbilewicz, B. (2007). *Cell fusion during development*. Trends in Cell Biology 17(11): 537-546

Pileri, P., Y. Uematsu, S. Campagnoli, G. Galli, F. Falugi, R. Petracca, A. J. Weiner, M. Houghton, D. Rosa, G. Grandi, and S. Abrignani. (1998). *Binding of hepatitis C virus to human CD81*. Science 282**:**938-941

Poppe, K., Glinoer, D., Van Steirteghem, A., Tournaye, H., Devroey, P., Schiettecatte, J. and B. Velkeniers (2002). *Thyroid dysfunction and autoimmunity in infertile women.* Thyroid 12(11):997-1001.

Primakoff, P. and D. G. Myles (2007). *Cell-cell membrane fusion during mammalian fertilization*. FEBS Lett 581(11): 2174-2180.

Rizo, J. and Rosenmund, C. (2008). *Synaptic vesicle fusion*, Nat Struct Mol Biol, 15(7): 665-674

Rubinstein, E., A. Ziyyat, M. Prenant, E. Wrobel, J. P. Wolf, S. Levy, F. Le Naour and C. Boucheix (2006). *Reduced fertility of female mice lacking CD81*. Dev Biol 290(2): 351-358.

Rubinstein, E., A. Ziyyat, J. P. Wolf, F. Le Naour and C. Boucheix (2006). *The molecular players of sperm-egg fusion in mammals*. Semin Cell Dev Biol 17(2): 254-263.

Runge, K. E., J. E. Evans, Z. Y. He, S. Gupta, K. L. McDonald, H. Stahlberg, P. Primakoff and D. G. Myles (2007). *Oocyte CD9 is enriched on the microvillar membrane and required for normal microvillar shape and distribution*. Dev Biol 304(1): 317-325.

Sachdev, M., A. Mandal, S. Mulders, L. C. Digilio, S. Panneerdoss, V. Suryavathi, E. Pires, K. L. Klotz, L. Hermens, M. B. Herrero, C. J. Flickinger, M. van Duin and J. C. Herr (2012). *Oocyte specific oolemmal SAS1B involved in sperm binding through intra-acrosomal SLLP1 during fertilization*. Dev Biol 363(1): 40-51.

Sebkova, N., Ded, L., Vesela, K., and Dvorakova-Hortova, K., *Progres sof sperm Izumo relocation during spontaneous acrosome reaction*, PLoS One, submitted

Stein, K. K., P. Primakoff and D. Myles (2004). *Sperm-egg fusion: events at the plasma membrane*. J Cell Sci 117(Pt 26): 6269-6274.

Tachibana, I., and M.E. Hemler. (1999). *Role of transmembrane 4 superfamily (TM4SF) proteins CD9 and CD81 in muscle cell fusion and myotube maintenance*. J. Cell Biol. 146*:*893–904.

Takeda, Y., Tachibana, I., Miyado, K., Kobayashi, M., Miyazaki, T., Funakoshi, T., Kimura, H., Yamane, H., Saito, Y., Goto, H., Yoneda, T., Yoshida, M., Kumagai, T.,

Osaki, T., Hayashi, S., Kawase, I. and E. Mekada (2003). *Tetraspanins CD9 and CD81 function to prevent the fusion of mononuclear phagocytes***.** J Cell Biol. 2003 Jun 9;161(5):945-56.

Talbot, P., B. D. Shur and D. G. Myles (2003). *Cell adhesion and fertilization: steps in oocyte transport, sperm-zona pellucida interactions, and sperm-egg fusion*. Biol Reprod 68(1): 1-9.

Tash, J. S. and A. R. Means (1983). *Cyclic adenosine 3',5' monophosphate, calcium and protein phosphorylation in flagellar motility*. Biol Reprod 28(1): 75-104.

Taylor, M. V. (2002). *Muscle differentiation: how two cells become one*. Curr Biol 12(6): R224-228.

Veselá, K. (2012). *Dynamika akrozomální reakce při vnitrodruhové kompetici spermií hlodavců*. Master´s Thesis, Charles University in Prague, Faculty of Science

Vesterlund, L., V. Töhönen, O. Hovatta and J. Kere (2011). *Co-localization of neural cell adhesion molecule and fibroblast growth factor receptor 2 in early embryo development*. Int J Dev Biol 55(3): 313-319.

Waterhouse, R., C. Ha and G. S. Dveksler (2002). *Murine CD9 is the receptor for pregnancy-specific glycoprotein 17*. J Exp Med 195(2): 277-282.

Weber, T., B. V. Zemelman, J. A. McNew, B. Westermann, M. Gmachl, F. Parlati, T. H. Söllner, and J. E. Rothman (1998). SNAREpins: minimal machinery for membrane fusion. Cell. 92:759–772.

White, J. M., S. E. Delos, M. Brecher and K. Schornberg (2008). *Structures and mechanisms of viral membrane fusion proteins: multiple variations on a common theme*. Crit Rev Biochem Mol Biol 43(3): 189-219.

Yamagata, K., T. Nakanishi, M. Ikawa, R. Yamaguchi, S. B. Moss and M. Okabe (2002). *Sperm from the calmegin-deficient mouse have normal abilities for binding and fusion to the egg plasma membrane*. Dev Biol 250(2): 348-357.

Yanagimachi, R. *(*1988*). Sperm-egg fusion. In* Current Topics in Membranes and Transport*, Vol.* 32 (ed. F. Bronner), pp. 3-43. San Diego, CA: Academic Press.

Yanagimachi, R. and Noda, Y.D. (1970). *Physiological changes in the post-nuclear cap region of mammalian spermatozoa: A necessary preliminary to the membrane fusion between sperm and egg cells*. Journal of Ultrastructure Research 31:486-493.

Zhu, X. and J. P. Evans (2002). *Analysis of the roles of RGD-binding integrins, alpha(4)/alpha(9) integrins, alpha(6) integrins, and CD9 in the interaction of the fertilin beta (ADAM2) disintegrin domain with the mouse egg membrane*. Biol Reprod 66(4): 1193-1202.

Zoller M. (2009). *Tetraspanins: push and pull in suppressing and promoting metastasis*. Nat Rev Cancer 9(1): 40-55

Zuccotti, M., A. Piccinelli, P. Giorgi Rossi, S. Garagna and C. A. Redi (1995). *Chromatin organization during mouse oocyte growth*. Mol Reprod Dev 41(4): 479-485.

List of attachments

Enclosed CD

CD contains two short lapsed videos.

No.1 is spatial model of FCRL3 distribution in the fixed mouse egg. FCRL3 is labelled by green signal, whereas chromatin is labelled in blue by DAPI.

No.2 is a 3-dimensional model of FCRL3 distribution in the fixed mouse egg. The red cyan 3D glasses are needed for viewing. FCRL3 is labelled by green signal, whereas chromatin is labelled in blue by DAPI